Etude du rôle du récepteur nucléaire FXRα dans la physiologie et la physiopathologie testiculaire

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LISTE DES PUBLICATIONS

Une partie de ces travaux a fait l'objet d'articles en préparation ou en cours de publication :

Article I

Bile acids impact urogenital tract* via *PXR signaling pathways in FXRα deficient mice.

Martinot E, Baptissart M, Vega A, Sedes L, Rouaisnel B, Saru JP, de Haze A, Lobaccaro JMA, Baron S, Schoonjans K, Caira F, Volle DH

En préparartion

Article II

Bile acid-FXRα pathways regulate male sexual maturation in mice.

Martinot E, Baptissart M, Vega A, Sedes L, Rouaisnel B, Baron S, Schoonjans K, Caira F, Volle DH

En révision, Oncotarget

Article III

Identification of multiple role of the bile acid nuclear receptor FXRα in mouse testis.

Martinot E, Sedes L, Baptissart M, Rouaisnel B, Saru JP, de Haze A, Thibault-Carpentier C, Keime C, Lobaccaro JMA, Baron S, Benoit G, Schoonjans K, Caira F, Volle DH

En préparation

Les articles suivants sont présentés en annexe :

Annexe I

Is spermiogenesis the critical step for answering biomedical issues ? *(review)*

Baptissart M, Vega A, Martinot E, Volle DH

Spermatogenesis, 2013

Annexe II

Farnesoid X receptor alpha: a molecular link between bile acids and steroid signaling ? *(review)*

Baptissart M, Vega A, Martinot E, Baron S, Lobaccaro JMA, Volle DH

Cellular and Molecular Life Sciences, 2013

Annexe III

Bile Acids Alter Male Fertility Through G-Protein-Coupled Bile Acid Receptor 1 Signaling Pathways in Mice.

Baptissart M, Vega A, Martinot E, Pommier AJ, Houten SM, Marceau G, de Haze A, Baron S, Schoonjans K, Lobaccaro JMA, Volle DH

Hepatology, 2014

Annexe IV

Hepatotoxicity induced by neonatal exposure to diethylstilbestrol is maintained throughout adulthood via the nuclear receptor SHP.

Vega A, Baptissart M, Martinot E, Saru JP, Baron S, Schoonjans K, Volle DH

Expert Opinion, 2014

Annexe V

Identification of the link between the hypothalamo-pituitary axis and the testicular orphan nuclear receptor NR0B2 in adult male mice.

Vega A, Martinot E, Baptissart M, de Haze A, Saru JP, Baron S, Caira F, Schoonjans K, Lobaccaro JMA, Volle DH

Endocrinology, 2014

Annexe VI

Bile acid alters male mouse fertility in metabolic syndrome context.

Vega A*, Martinot E*, Baptissart M, De Haze A, Vaz F, Kulik W, Damon-Soubeyrand C, Baron S, Caira F, Volle DH

Plos One, 2015

RESUME

Fxrα est le récepteur nucléaire des acides biliaires, exprimé majoritairement dans le foie, l'intestin, les reins et les glandes surrénales. L'intérêt pour ce dernier est devenu croissant au cours des dernières années, de part le rôle central qu'il joue dans le contrôle de l'homéostasie du cholestérol, des acides biliaires, des triglycérides ou encore du glucose.

Plus récemment, Fxrα ainsi que ses ligands, les acides biliaires, ont été localisés dans le testicule, soulevant la question du rôle potentiel de Fxrα dans cet organe, et plus généralement dans la fonction de reproduction mâle. Mais les études menées à ce sujet restent jusqu'à présent peu nombreuses, et focalisées sur son implication dans le contrôle du métabolisme des stéroïdes : l'activation *in vivo* de Fxrα par un agoniste synthétique conduit ainsi chez l'adulte à court terme à une répression de la stéroïdogenèse.

Outre son rôle dans le contrôle de l'activité endocrine des cellules de Leydig, l'impact de l'activation *in vivo* de Fxrα sur la physiologie plus globale du testicule n'a jamais été abordé à ce jour. De telles études seraient pourtant pertinentes étant donné que Fxrα est ciblé pour le traitement de pathologies métaboliques telles que la dyslipidémie ou le diabète.

Dans ce contexte, l'objectif de ce travail de thèse était d'étudier le rôle de Fxrα dans la physiologie et la patho-physiologie du testicule, en s'appuyant sur l'analyse d'un modèle murin dont le gène codant Fxrα a été invalidé. Nos résultats démontrent que : 1) la perte de Fxrα prédispose le testicule à une sur-mortalité des cellules germinales dans un contexte pathologique de cholestase ; 2) la sur-activation de la signalisation Fxrα au cours de la puberté conduit à un défaut de la différenciation germinale, associée à une altération de la fonction endocrine du testicule ; 3) outre la régulation de la stéroïdogenèse dans les cellules de Leydig, Fxrα participe au contrôle des fonctions sertoliennes et de la prolifération et / ou différenciation des cellules germinales souches.

L'ensemble de ces données définissent Fxrα comme un nouvel acteur impliqué dans le contrôle de la physiologie testiculaire et devraient être prises en considération quant-à l'utilisation de molécules agonistes et / ou antagonistes de Fxrα dans le cadre du traitement de pathologies métaboliques.

Mots clés : Fxrα, testicule, stéroïdogenèse, apoptose et différenciation germinale, cellules germinales souches.

Abstract

Fxrα is the bile acid nuclear receptor, predominantly expressed in liver, intestine, kidney and adrenal glands. In recent years, interest in Fxrα has been increasing due to its central role in the control of cholesterol, bile acids, triglycerides or glucose homeostasis.

More recently, Fxrα and its ligands, bile acids, have been detected in the testis pointing out its potential involvement in this tissue and more widely in the male reproductive functions. However, the few studies on this topic focused essentially on Fxrα involvement in the control of steroids metabolism. Indeed, activation of Fxrα *in vivo* with a synthetic agonist leads to short-term steroidogenesis repression in the adult.

In vivo the impact of alteration of Fxrα signaling on the global testis physiology has never been explored so far. Such studies would be pertinent considering that Fxrα is a target for the treatment of metabolic diseases such as dyslipidemia or diabetes.

In this context, the aim of my work was to study the implication of Fxrα in testis physiology and physiopathology by analyzing a knock out mouse model for Fxrα. Our results show that: 1) the loss of Fxrα increase germ cell mortality in the testis in a disease context of cholestasis ; 2) over-activation of Fxrα signaling during puberty leads to germ cell differentiation defects, associated with an alteration of testis endocrine function ; 3) besides steroidogenesis control in Leydig cell, Fxrα is involved in Sertoli cell functions and spermatogonial stem cell proliferation and/or differentiation.

Taken together, these data define Fxrα as a new actor involved in the control of testis physiology, and should be taken into consideration regarding the use of Fxrα agonistic or antagonistic ligands for the treatment of metabolic diseases.

Key words : Fxrα, testis, steroidogenesis, germ cell apoptosis and differenciation, spermatogonial stem cells.

ABREVIATIONS

ABC	*ATP Binding Cassette*
ABP	*Androgen Binding Protein*
AceCS	*Acetyl-CoA Synthetase*
AF	*Activating Function*
AKR	*Aldo-Keto Reductase*
AMH	*Anti-Müllerian Hormone*
AMPc	Adénosine Monophosphate cyclique
Apo	*Apolipoprotein*
AR	*Androgen Receptor*
ASBT	*Apical Sodium-dependent Bile acid Transporter*
BACS	*Bile Acid Coenzyme A Synthase*
BAAT	*Bile Acid-coA : amino acid N-acyltransferase*
BHT	Barrière Hémato-Testiculaire
BSEP	*Bile Salt Export Pump*
BSH	*Bile Salt Hydrolase*
CA	*Cholic Acid*
CAMK	*Calmodulin Kinase*
CAR	*Constitutive Androstan Receptor*
CCNA1	*Cyclin A1*
CDCA	*Chenodeoxycholic Acid*
CDKI	*Cyclin-Dependent Kinase Inhibitor*
COUP-TF	*Chicken Ovalbumin Upstream Promoter-Transcription Factor*
CREB	*cAMP Response Element Binding Protein*
CYP	*Cytochrome*
DAX-1	*Dosage-sensitive sex reversal, Adrenal hypoplasia critical region, on chromosome X, gene 1*
DAZL	*Deleted in Azoospermia-Like*
DBD	*DNA-Binding Domain*
DCA	*Deoxycholic Acid*
DHH	*Desert Hedgehog*
DHT	Dihydrotestostérone
DMC1	*Disrupted Meiotic gene 1*
DMRT1	*Doublesex-related and Mab-3 Related Transcription factor 1*
DR	*Direct Repeat*
E	stade Embryonnaire
E2	*oestradiol*
EAR	*ERBA-related gene*
E-Cadh	*E-Cadherin*
ECDCA	*Ethyl-Chenodeoxycholic Acid*
eFABP	epidermal Fatty-Acid-Binding Protein
EGF	*Epidermal Growth Factor*
ELMO1	*Engulfment and cell Motility 1*
ER	*Everted Repeat*
ERM	*Ets Related Molecule*
ERK	*Extracellular signal-Related Kinase*
FGF	*Fibroblast Growth Factor*
FGFR	*Fibroblast Growth Factor Receptor*

FOXO1	*Forkhead box protein O1*
FSH	*Follicle Stimulating Hormone*
FSH-Rc	*FSH-Receptor*
FXR	*Farnesoid X Receptor*
GDNF	*Glial cell Derived Neurotrophic Factor*
GFRα1	*GDNF Family Receptor alpha 1*
GLUT	*Glucose Transporter*
GnRH	*Gonadotropin Releasing Hormone*
GnRH-R	*GnRH-Receptor*
G-6-Pase	*Glucose-6-Phosphatase*
GPBAR1	*G Protein-coupled Bile Acid Receptor 1*
GPER	*G Protein-coupled Estrogen Receptor*
GPR30	*G Protein-coupled Receptor 30*
GST	*Glutathione S-Transferase*
HDL	*High-Density Lipoprotein*
HMG-CoA	*3-Hydroxy-3-Methylgutaryl-Coenzyme A*
HMOX	*Heme Oxygenase*
HNF4α	*Hepatocyte Nuclear Factor 4α*
HSD	*Hydroxysteroid Dehydrogenase*
HSP	*Heat Shock Protein*
HRE	*Hormone Response Element*
IBABP	*Ileal Bile Acid Binding Protein*
ICP	*Intrahepatic Cholestasis of Pregnancy*
IGF	*Insulin-like Growth Factor*
INSL3	*Insulin-Like 3*
IR	*Inverted Repeat*
JNK	*Jun N-terminal Kinase*
KLF4	*Krüppel-Like Factor 4*
LBD	*Ligand Binding Domain*
LCA	*Lithocholic Acid*
LH	*Luteinizing Hormone*
LH-Rc	*LH-Receptor*
LPL	*Lipoprotein Lipase*
LRH-1	*Liver Receptor Homolog-1*
LXR	*Liver X Receptor*
MafG	*v-maf musculoaponeurotic fibrosarcoma oncogene homolog G*
MAPK	*Mitogen-Activated Protein Kinase*
MDR	*Multidrug Resistance*
ME	*Malic Enzyme*
MRP	*Multidrug Resistance-associated Protein*
N-CoR	*Nuclear receptor Co-Repressor*
NGFIB	*Nerve Growth Factor-Induced clone B*
Ngn-3	*Neurogenin-3*
NLS	*Nuclear Localisation Signal*

NQO	*NRH : Quinone Oxidoreductase*
NR	*Nuclear Receptor*
NTCP	*Na^+-Taurocholate Cotransport Polypeptide*
OATP	*Organic Anion Transporting Polypeptide*
8-OHDG	*8-Hydroxy-2'-Deoxyguanosine*
OST	*Organic Solute Transporter*
PBR	*Peripheral-type Benzodiazepine Receptor*
PCN	*Pregnenolone-16α-Carbonitrile*
PDGF	*Plateled-Derived Growth Factor*
PEPCK	*Phosphoenolpyruvate Carboxykinase*
PGCs	*Primordial Germ Cells*
PI3-K	*Phosphatidylinositol 3-Kinase*
PKA	*Protein Kinase A*
PKB	*Protein Kinase B*
PKC	*Protein Kinase C*
PLC	*Phospholipase C*
PLZF	*Promyelocytic Leukemia Zinc Finger protein*
PPAR	*Peroxisome Proliferator-Activated Receptor*
Prm	*Protamin*
PXR	*Pregnane X Receptor*
PXT1	*Peroxisomal testis-specific 1*
RAR	*Retinoic Acid Receptor*
RSPO1	*R-Spondin-1*
RXR	*Retinoid X Receptor*
RIP14	*RXR Interacting Protein 14*
SCD-1	*Stearoyl-CoA Desaturase-1*
SCF	*Stem Cell Factor*
SF-1	*Steroidogenic Factor-1*
SHBG	*Sex Hormone Binding Globulin*
SHP	*Small Heterodimer Partner*
SIRT1	*Sirtuin 1*
SLF	*Steel Factor*
SMRT	*Silencing Mediator for Retinoic acid and Thyroid hormone receptors*
SNP	*Single Nucleotide Polymorphism*
SOX9	*Sry-related HMG box 9*
SR-B1	*Scavenger Receptor class B, member 1*
SREBP-1c	*Sterol Regulatory Element Binding Protein-1c*
Sry	*Sex determination Region on Y chromosome*
SSC	*Spermatogonial Stem Cell*
STAR	*Steroidogenic Acute Regulatory Protein*
STAT	*Signal Transducer And Activator Of Transcription*
STRA8	*Stimulated by Retinoic Acid gene 8*
SULT	*Sulfotransferase*
SYCP	*Synaptonemal Complex Protein*
TAF4B	TBP-Associated Factor 4B
TBARS	*Thiobarbituric Acid Reactive Substances*
TBP	TATA box-Binding Protein

T_3	*Triiodothyronine*
TeBG	*Testosterone Binding-Globulin*
TGF	*Transforming Growth Factor*
TNP	*Transition Nuclear Protein*
UDCA	*Ursodeoxycholic Acid*
UGT	*Uridine Glucuronosyltransferase*
VDR	*Vitamin D3 Receptor*
VLDL	*Very Low-Density Lipoprotein*
WNT4	*Wingless-type MMTV integration site family, member 4*
ZO	*Zonula Occludens*

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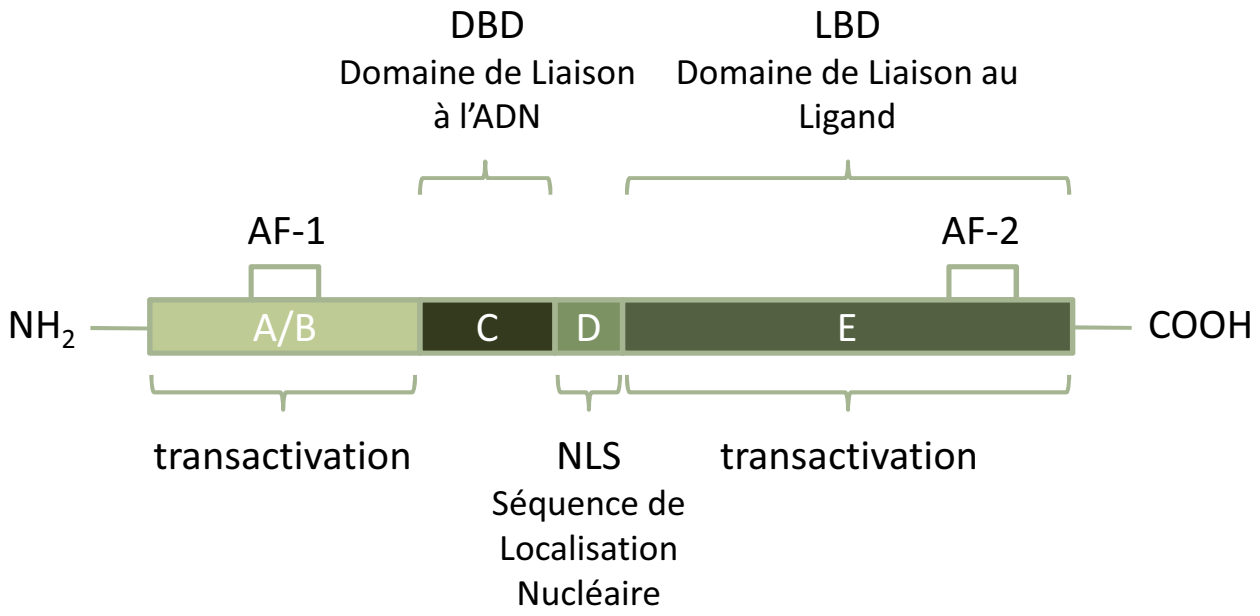


Figure 1 : Structure des récepteurs nucléaires. Tous les membres de la superfamille des récepteurs nucléaires partagent une structure commune composée de quatre domaines fonctionnels distincts : un domaine A/B de transactivation, un domaine C de liaison à l'ADN, un domaine charnière D et un domaine E de liaison au ligand. Les domaines AF-1 et -2 permettent l'interaction du récepteur avec les co-facteurs transcriptionnels.
AF : Activating Function ; DBD : DNA Binding Domain ; LBD : Ligand Binding Domain ; NLS : Nuclear Localisation Signal. Adapté d'après Aranda and Pascual, 2001.

Partie 1 : Farnesoid X Receptor α (Fxrα)

A. La super-famille des récepteurs nucléaires

1. Généralités

Les récepteurs nucléaires forment une super-famille de facteurs de transcription dépendants du ligand. Le clonage du premier de ces récepteurs (celui des glucocorticoïdes) date de 1985 (1). Depuis, de nombreuses études ont conduit, et conduisent encore, à l'identification de nouveaux membres de cette famille de récepteurs (qui en comprend aujourd'hui 48 chez l'Homme et la souris), sur la base d'une homologie de séquence qui existe entre eux tous. Les récepteurs nucléaires contrôlent la transcription de gènes cibles impliqués dans des fonctions variées, telles que le développement, la différenciation cellulaire, la reproduction et le métabolisme général.

2. Structure des récepteurs nucléaires

Les membres de la famille des récepteurs nucléaires sont caractérisés par une structure très conservée, subdivisée en 4 domaines fonctionnels (2) (**Figure 1**) :

La partie N-terminale (domaine A/B) est la plus variable en terme de taille et de séquence protéique. Elle comprend un domaine d'activation de la transcription *Activating Function-1* (AF-1) responsable de l'interaction du récepteur avec des co-activateurs et des co-répresseurs, indépendamment de la présence du ligand. L'intervention de ces co-facteurs est indispensable à l'activité transcriptionnelle du récepteur.

Le domaine central (C) est le plus conservé au sein du groupe, et contient le site de liaison à l'ADN (ou *DNA-Binding Domain*, DBD). Il comprend deux structures en doigt de zinc impliquées d'une part dans la liaison à l'ADN, et d'autre part dans la dimérisation des récepteurs. Ces derniers modulent en effet la transcription de leurs gènes cibles, *via* leur fixation sur des séquences promotrices spécifiques appelées éléments de réponse aux hormones (*Hormone Response Elements*, HREs). Les HREs sont en général composés de deux hémi-sites de 6 bases chacun, reliés par un espacement de 0 à 5 bases, et organisés en répétitions directes (*Direct Repeat*, DR), inversées (*Inverted Repeat*, IR) ou en miroir (*Everted Repeat*, ER). Les récepteurs nucléaires se fixent sur ces éléments de réponse sous forme d'homodimères, d'hétérodimères ou de monomères (3) grâce à la présence de ce domaine.

Le domaine D est une région charnière flexible reliant le DBD au LBD (*Ligand Binding Domain).* Il permettrait la rotation du LBD pour faciliter la fixation des dimères sur des éléments de réponse de type DR ou IR. Il contient par ailleurs le signal de localisation nucléaire (*Nuclear Localisation Signal*,

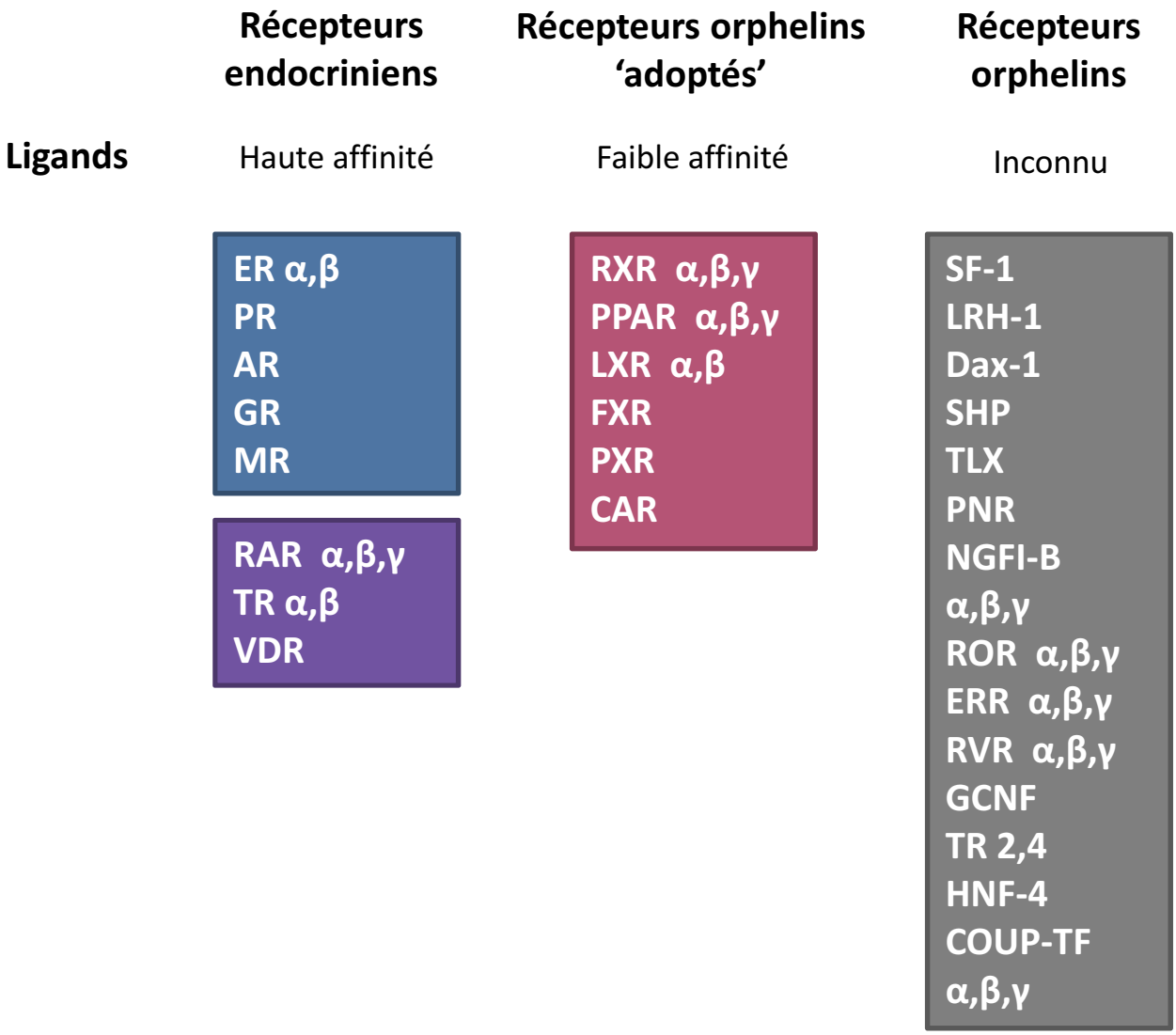


Figure 2 : Classification des 48 récepteurs nucléaires chez les mammifères en fonction de leur ligand. D'un point de vue fonctionnel, on distingue trois classes de récepteurs nucléaires : les récepteurs endocriniens lient leur ligand, avec une haute affinité ; les récepteurs nucléaires orphelins ''adoptés'' ont une affinité relative faible pour leurs ligands ; les récepteurs nucléaires orphelins n'ont à ce jour aucun ligand identifié.
AR : Androgen Receptor ; CAR : Constitutive Androstane Receptor ; COUP-TF : Chicken Ovalbumin Upstream Promoter-Transcription Factor ; DAX-1 : Dosage-sensitive sex reversal-Adrenal hypoplasia congenita critical region on the X chromosome, gene 1 ; ER : Estrogen Receptor ; ERR : Estrogen-Related Receptor ; FXR : Farnesoid X Receptor ; GCNF : Germ Cell Nuclear Factor ; GR : Glucocorticoid Receptor ; HNF-4 : Hepatocyte Nuclear Factor-4 ; LRH-1 : Liver Receptor Homolog-1 ; LXR : Liver X Receptor ; MR : Mineralocorticoid Receptor ; NGFI-B : Nerve Growth Factor IB-like receptor ; PNR : Photoreceptor-specific Nuclear Receptor ; PPAR : Peroxisome Proliferator-Activated Receptor ; PR : Progesterone Receptor ; PXR / SXR : Pregnane X Receptor / Steroid and Xenobiotic Receptor ; RAR : Retinoic Acid Receptor ; ROR : RAR-related Orphan Receptor ; RXR : Retinoid X Receptor ; SF-1 : Steroidogenic Factor-1 ; SHP : Small Heterodimer Partner ; TLX : Tailless homolog ; TR : Thyroid hormone Receptor ; TR2,4 : Testicular orphan Receptor 2,4 ; VDR : Vitamin D Receptor. Adapté d'après Chawla *et al.* 2001.

NLS) qui assure le transfert du récepteur dans le noyau. Il contacte enfin des co-répresseurs en l'absence de ligand maintenant la transcription des gènes cibles du récepteur inactive (4)-(5).

Le domaine E correspond au domaine de liaison au ligand (ou LBD) qui assure la spécificité et la sélectivité de la réponse physiologique. Il contient également un deuxième domaine d'activation de la transcription (AF-2).

3. Classification des récepteurs nucléaires

D'un point de vue fonctionnel, on distingue trois classes de récepteurs nucléaires : les récepteurs endocriniens ayant un ligand de forte affinité, les récepteurs nucléaires orphelins ''adoptés'' possédant un ligand de faible affinité, et les récepteurs nucléaires orphelins (6) (**Figure 2**).

3.1. Les récepteurs endocriniens

Les récepteurs endocriniens ont pour ligand les hormones stéroïdes. Il en existe 5 classes : les androgènes, les œstrogènes, les progestines, les glucocorticoïdes et les minéralcorticoïdes. En l'absence de ligand, ces récepteurs sont complexés à des protéines chaperonnes de type *Heat Shock Protein* (Hsp) qui les séquestrent dans le cytoplasme, les rendant ainsi inactifs. La liaison du ligand entraîne le départ des protéines Hsp, la translocation nucléaire du récepteur et sa dimérisation. Les récepteurs de cette classe se lient spécifiquement sous forme d'homodimère sur le promoteur de leurs gènes cibles pour en favoriser l'expression grâce au recrutement de co-activateurs transcriptionnels.

En dehors de ce mode d'action dit ''génomique'', certaines de ces hormones stéroïdes sont capables d'agir par d'autres mécanismes moléculaires plus rapides dits ''non génomiques''. De telles voies de signalisation ont été identifiées pour les androgènes et les œstrogènes. Les androgènes exercent en effet en partie leur action par l'activation d'un récepteur membranaire couplé aux protéines G, entraînant, *via* la signalisation de la phospholipase C (PlC), un influx calcique. Leur récepteur nucléaire *Androgen Receptor* (Ar) est par ailleurs capable d'interagir et d'activer la protéine kinase Src, déclenchant une cascade de phosphorylations impliquant le récepteur du facteur de croissance *Epidermal Growth Factor* (Egf) et la voie des *Mitogen-Activated Protein Kinase* (Mapk), aboutissant à l'activation du facteur de transcription *cAMP Response Element Binding Protein* (Creb) (7). L'action des œstrogènes peut également conduire à l'activation de la voie des Mapk et à la mobilisation du calcium intracellulaire par l'intermédiaire d'un récepteur membranaire couplé aux protéines G nommé *G Protein-coupled Estrogen Receptor* ou *G Protein-coupled Receptor 30* (Gper ou Gpr30) (8)-(9). La mise en jeu de ces voies de signalisation permet aux stéroïdes d'étendre la portée de leur

action, en modulant l'expression d'un panel de gènes bien plus large que celui sous l'influence de leur seule action génomique, et leur confère une plus grande rapidité d'action.

3.2. Les récepteurs adoptés

Les récepteurs nucléaires orphelins ''adoptés'', seraient fixés en permanence sur leurs éléments de réponse, et associés à des co-répresseurs transcriptionnels maintenant la chromatine dans un état non permissif vis-à-vis de la transcription (10)-(11). La fixation du ligand provoque un changement de conformation du récepteur permettant le relargage des co-répresseurs, et le recrutement de co-activateurs à l'origine de l'activation de la transcription des gènes cibles. Ces récepteurs agissent majoritairement sous la forme d'un hétérodimère obligatoire avec le récepteur de l'acide rétinoïque 9-*cis, Retinoid X Receptor* (Rxr, Nr2b1) (3). Cependant des données montrent qu'ils sont aussi capables d'agir sous forme d'homodimères ou de monomères (12). Cette classe de récepteurs nucléaires comprend notamment les membres *Liver X Receptors* (Lxrα,β, Nr1h3 et Nr1h2) et *Peroxisome Proliferator-Activated Receptors* (Pparα,β,γ, Nr1c1, Nr1c2 et Nr1c3) impliqués dans le contrôle du métabolisme lipidique (13)-(14) et (15).

3.3. Les récepteurs orphelins

Les récepteurs orphelins sont des protéines dont la structure présente une forte identité avec les autres membres de la famille des récepteurs nucléaires, mais dont les ligands naturels n'ont pas encore été identifiés.

C'est ainsi le cas des récepteurs *Steroidogenic Factor-1* (Sf-1, Nr5a1) et *Liver Receptor Homolog-1* (Lrh-1, Nr5a2), hautement apparentés l'un à l'autre (16)-(17). Sf-1 joue un rôle clé dans la détermination sexuelle au cours du développement, et dans la régulation de la stéroïdogenèse gonadique et surrénalienne chez l'adulte. Lrh-1 est quant-à lui impliqué dans le contrôle de l'homéostasie du cholestérol et des acides biliaires (18). Plusieurs études indépendantes suggèrent néanmoins que les phospholipides pourraient jouer le rôle de ligand pour ces deux récepteurs (19)-(20)-(21).

Dosage-sensitive sex reversal, Adrenal hypoplasia critical region, on chromosome X, gene 1 (Dax-1, Nr0b1) et *Small Heterodimer Partner* (Shp, Nr0b2) sont des membres atypiques de la famille des récepteurs nucléaires, dépourvus de domaine de liaison à l'ADN (22)-(23). Ils sont néanmoins capables d'interagir avec d'autres récepteurs nucléaires, inhibant ainsi leur activité transcriptionnelle (22)-(24)-(25)-(26)-(27). La mutation de Dax-1 est à l'origine du développement de l'hypoplasie surrénalienne congénitale chez l'Homme (23). Des études ont par la suite démontré son implication dans la détermination sexuelle et le développement des gonades (28)-(29). Shp est principalement

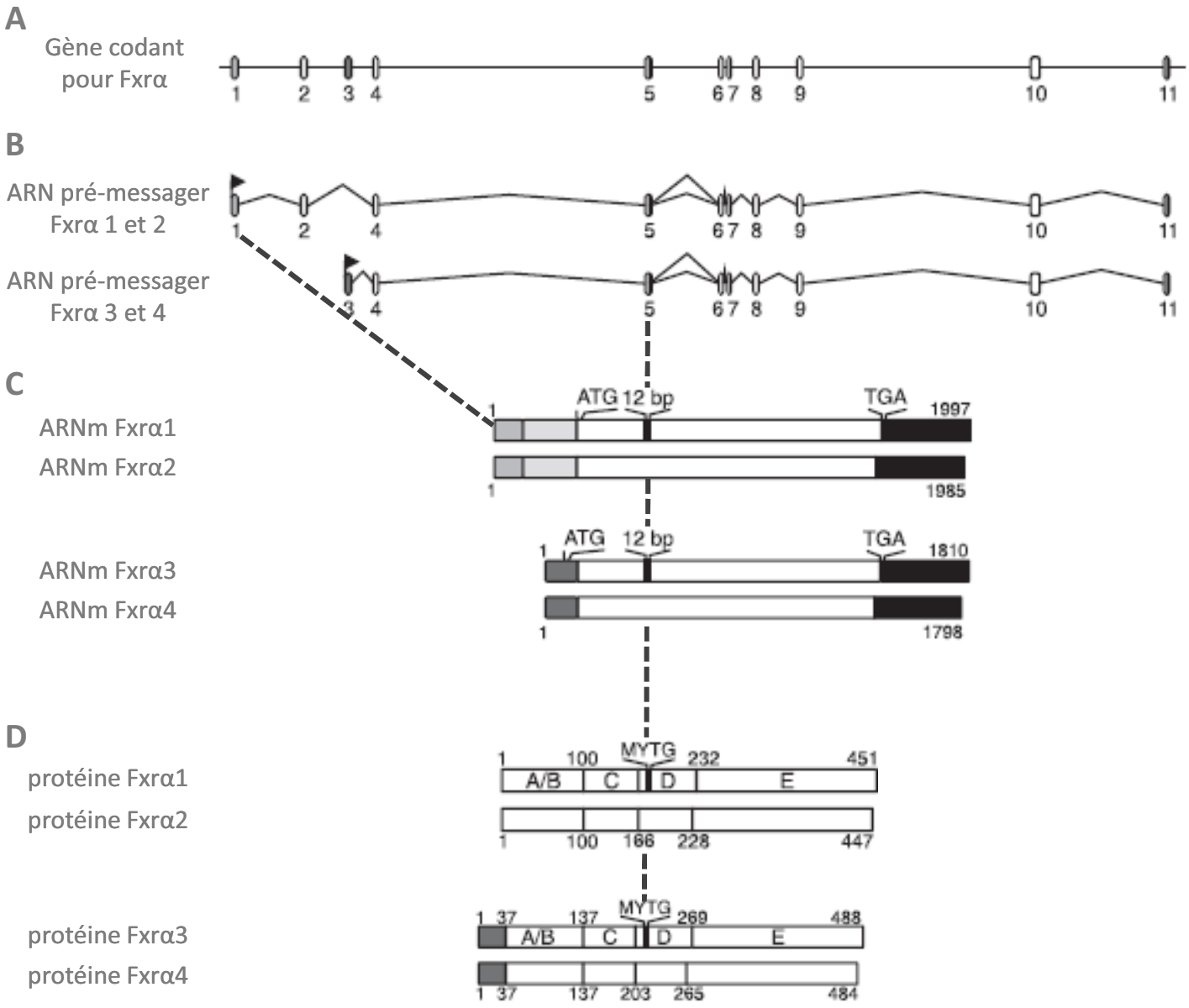


Figure 3 : Structure du gène murin codant les 4 isoformes de Fxrα. A. Le gène murin codant pour Fxrα comprend 11 exons et 10 introns. **B.** Représentation schématique des ARN pré-messagers des isoformes de Fxrα. Les promoteurs alternatifs localisés au niveau des exons 1 et 3 sont à l'origine respectivement des isoformes Fxrα1 et 2 et Fxrα3 et 4. **C.** L'épissage alternatif entre les exons 5 et 6 produit deux types d'isoformes contenant (Fxrα1 et 3) ou pas (Fxrα2 et 4) une insertion de 12 paires de base dans la séquence nucléotidique de leur messager. **D.** Ces évènements transcriptionnels se traduisent au niveau protéique par la présence d'une séquence additionnelle de 37 acides aminés dans la région N-terminale (domaine A/B) des isoformes Fxrα3 et 4, et de 4 acides aminés (MYTG) au niveau de la région charnière (domaine D) des isoformes Fxrα1 et 3. Les domaines de liaison à l'ADN (C) et au ligand (E) sont représentés. Adapté d'après Lefebvre *et al.* 2009.

connu pour son rôle dans le contrôle de l'homéostasie des acides biliaires, des triglycérides et du glucose (30)-(31)-(32), et a été plus récemment impliqué dans la régulation de la physiologie testiculaire (33).

B. Farnesoid X Receptor α

1. Identification du récepteur nucléaire Fxrα

En 1995, Seol *et al.* (34) ont isolé, grâce au système du double hybride chez la levure, des ADNc de foie de souris, codant pour une protéine capable d'interagir spécifiquement avec le domaine de liaison à l'ADN du récepteur nucléaire RXRα humain. Les transcrits de cette protéine, nommée *Rxr Interacting Protein 14* (Rip14), ont été détectés dans le foie et les reins. La même année, Forman *et al* (35) ont cloné l'orthologue de Rip14 chez le rat. Son nom actuel de *Farnesoid X Receptor* (Fxr, Nr1h4) lui fut alors attribué du fait de son activation par de fortes concentrations de farnésol (un intermédiaire de la voie du mévalonate qui conduit notamment à la synthèse du cholestérol et des stéroïdes). Comme pour Rip14, son expression a initialement été retrouvée restreinte au foie, aux reins, ainsi qu'à l'intestin et aux glandes surrénales.

L'analyse de la séquence protéique de Fxrα a révélé une forte identité avec les membres de la super-famille des récepteurs nucléaires (34)-(35). Fxrα appartient à la classe des récepteurs nucléaires orphelins ''adoptés''. Il agit donc en association avec Rxr (34)-(35), avec lequel il forme un hétérodimère obligatoire permissif (35). Le complexe Rxr/Fxrα se fixe préférentiellement sur un élément de réponse de type IR-1, constitué de 2 copies de la séquence hexanucléotidique AGGTCA arrangées en motifs répétés inversés, et séparées par une base (36). Cette configuration en hétérodimère est souvent associée à une activation transcriptionnelle (37). Néanmoins, Fxrα peut également réprimer la transcription de ses gènes cibles en se fixant sous forme de monomère sur un élément de réponse négatif (12), ou en entrant en compétition avec un autre facteur de transcription pour la fixation à l'ADN (38).

2. Structure du gène et isoformes

Chez la souris, le gène codant Fxrα comprend 11 exons et 10 introns. Il donne naissance à 4 isoformes, issues de l'existence d'un promoteur et d'un site d'épissage alternatifs (**Figure 3**). Initialement baptisées Fxrα1, Fxrα2, Fxrβ1 et Fxrβ2 (39), ces isoformes ont été renommées Fxrα1, 2, 3 et 4, suite à l'identification de Fxrβ (Nr1h5) (40), un pseudogène chez les primates, mais un récepteur du lanostérol, un intermédiaire de la voie de synthèse du cholestérol, chez les autres mammifères.

Les promoteurs alternatifs localisés au niveau des exons 1 ou 3, régulent l'expression des transcrits des isoformes 1 et 2, ou 3 et 4, respectivement. Ces deux dernières possèdent 37 acides aminés supplémentaires au niveau de leur séquence amino-terminale par rapport aux isoformes 1 et 2. L'extrémité N-terminale étant le site de localisation du domaine d'activation transcriptionnelle AF-1, la capacité de recrutement des co-facteurs par le récepteur pourrait être variable en fonction des isoformes.

Il existe un site d'épissage alternatif entre les exons 5 et 6, à l'origine de la production de deux types d'isoformes de Fxrα, possédant (Fxrα1 et Fxrα3) ou non (Fxrα2 et Fxrα4) une séquence nucléotidique de 12 paires de base supplémentaires. Cet évènement d'épissage se traduit par l'addition de 4 acides aminés (MYTG) dans la séquence protéique, localisés au niveau de la séquence charnière. Ces acides aminés semblent diminuer l'affinité de liaison à l'ADN et la capacité transactivatrice des isoformes concernées (Fxrα1 et Fxrα3) pour certains gènes cibles *in vitro* (39). Ils pourraient ainsi être responsables d'une modification de la structure tri-dimensionnelle du récepteur, altérant sa fixation à l'ADN, ou son intéraction avec des co-facteurs transcriptionnels.

Il est à noter que la structure du gène et l'existence de ces 4 isosformes sont conservées chez plusieurs espèces dont l'Homme (41). De plus, outre leurs différences structurales et fonctionnelles, les 4 isoformes de Fxrα diffèrent également de part leur distribution tissulaire (39). Les quatre isoformes ont un niveau d'expression équivalent dans le foie ; les isoformes Fxrα1 et 2 sont faiblement exprimées dans l'iléon et les reins contrairement aux isoformes Fxrα3 et 4, et inversement dans les glandes surrénales.

3. Ligands de Fxrα

3.1. Les acides biliaires et leurs dérivés

Initialement, Fxrα a été montré comme étant activable par de fortes concentrations de farnésol chez le rat (35) ; mais en 1997, Zavacki *et al.* ont montré que ce n'était pas le cas pas de son orthologue murin (42). Des efforts ont dès lors été faits afin de découvrir le ligand physiologique de Fxrα. Deux ans plus tard, 3 équipes indépendantes ont identifié les acides biliaires, et leurs formes conjuguées, comme ligands de Fxrα (43)-(44)-(45).

Les acides biliaires sont le constituant majeur de la bile. Leur synthèse exclusivement hépatique, à partir du cholestérol, constitue la voie principale d'élimination de ce dernier. Elle conduit à la production des acides biliaires primaires (acides cholique (*Cholic Acid*, CA) et chénodéoxycholique (*Chenodeoxycholic Acid*, CDCA)) (46) qui seront convertis par la flore bactérienne intestinale en acides biliaires secondaires (acides déoxycholique (*Deoxycholic Acid*, DCA) et lithocholique

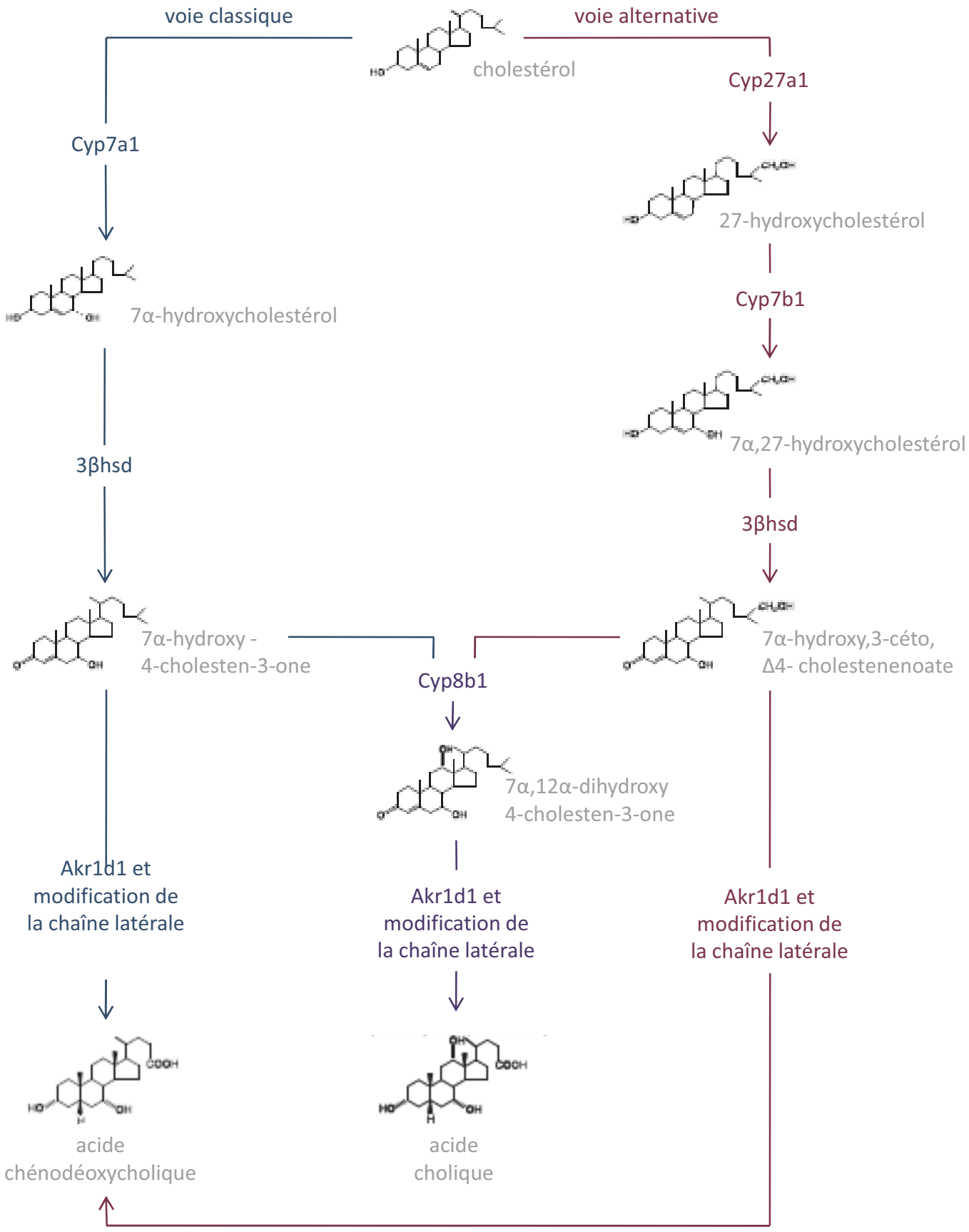


Figure 4 : Représentation schématique des voies de synthèse des acides biliaires. La synthèse hépatique des acides biliaires résulte d'une succession de modifications enzymatiques du noyau et de la chaîne latérale du cholestérol. Elle implique deux voies possibles, différant par les intermédiaires métaboliques et les acteurs enzymatiques qu'elles mettent en jeu : la voie classique et la voie alternative.
Cyp7a1 : cholesterol 7α-hydroxylase ; Cyp27a1 : sterol 27-hydroxylase ; Cyp7b1 : oxysterol 7α-hydroxylase ; 3ϐHsd : 3ϐ-Hydroxysteroid Dehydrogenase; Cyp8b1 : sterol 12α-hydroxylase ; Akr1d1 : Aldo-Keto Reductase 1 D1

(*Lithocholic Acid*, LCA) respectivement) (47). Le pool d'acides biliaires qui en résulte, possède des propriétés physico-chimiques variées permettant la solubilisation et la digestion des graisses alimentaires dans la lumière intestinale.

3.1.a. Synthèse hépatique

Le foie est à ce jour le seul organe à exprimer les 17 enzymes nécessaires à la synthèse des acides biliaires primaires (CA et CDCA). Elle résulte d'une succession de modifications enzymatiques du noyau et de la chaîne latérale du cholestérol, catalysées dans différents compartiments intra-cellulaires des hépatocytes (microsomes, mitochondries, cytosol, peroxysomes). Elle implique par ailleurs deux voies possibles, différant par les intermédiaires métaboliques et les acteurs enzymatiques qu'elles mettent en jeu : la voie classique (ou neutre) et la voie alternative (ou acide) (46) (**Figure 4**).

La voie classique est initiée par la conversion du cholestérol en 7α-hydroxycholestérol, par l'enzyme microsomale *cholesterol 7α-hydroxylase* (ou *Cytochrome P450 7a1,* Cyp7a1), enzyme limitante de cette voie de biosynthèse (48). La régulation précise de son expression est primordiale au maintien de l'homéostasie des acides biliaires et du cholestérol.

L'importance physiologique de l'activité de cette enzyme, et plus généralement de la synthèse des acides biliaires, est indéniable au vu du phénotype des souris dont le gène codant Cyp7a1 a été invalidé (49)-(50). 85% de ces souris meurent au cours des 3 premières semaines de vie en raison d'une insuffisance hépatique et d'un défaut d'absorption intestinale des lipides et des vitamines. L'analyse de ce modèle murin, et de données issues de patients déficients pour l'enzyme CYP7a1 (51), a permis d'estimer la contribution de la voie classique à la synthèse totale des acides biliaires à 75% chez la souris, et à plus de 90% chez l'Homme. En effet, les animaux survivants, commencent dès lors à synthétiser des acides biliaires par la voie alternative.

La voie alternative fait intervenir la conversion du cholestérol en 27-hydroxycholestérol, par l'enzyme mitochondriale *sterol 27-hydroxylase* (Cyp27a1). Cet oxystérol subit une hydroxylation supplémentaire catalysée par l'enzyme Cyp7b1 conduisant à la formation du 7α,27-dihydroxycholestérol (46).

Les intermédiaires 7α-hydroxylés synthétisés par les voies classique et alternative sont ensuite convertis dans leur forme 3-oxo,Δ^4 par la *3 beta-Hydroxysteroid Dehydrogenase type 7* (3β-Hsd7) (46). Le produit de cette étape peut alors s'engager dans deux routes enzymatiques distinctes afin de compléter la synthèse des acides biliaires. S'il est pris en charge par l'enzyme microsomale *sterol 12α-hydroxylase* (Cyp8b1), le produit final sera le CA. Dans le cas où cette 12α-hydroxylation n'a pas

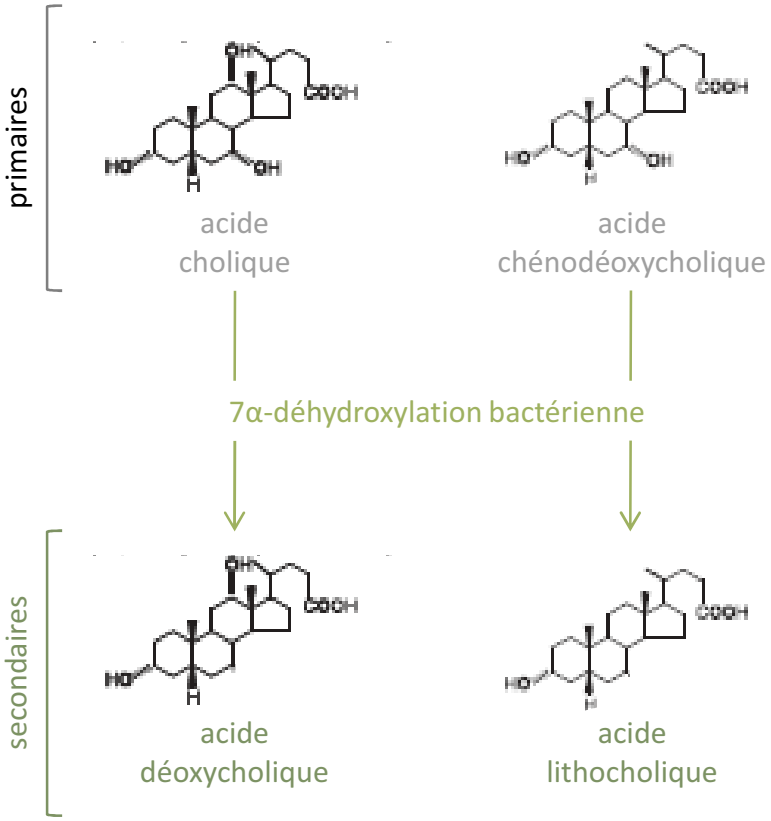


Figure 5 : Conversion des acides biliaires primaires en secondaires. Au cours de leur transit intestinal, les acides biliaires primaires cholique et chénodéoxycholique subissent une série de modifications structurales par la flore bactérienne, consistant majoritairement en leur déconjugaison et leur 7α-déhydroxylation, donnant naissance respectivement aux acides biliaires secondaires déoxycholique et lithocholique.

lieu, il en résultera du CDCA (46). L'activité de cette enzyme détermine ainsi le ratio CA/CDCA, et par conséquent les propriétés physicochimiques et biologiques du pool d'acides biliaires. Les intermédiaires 12α-hydroxylés, ainsi que ceux ayant échappés à l'activité de la Cyp8b1, sont tous soumis à l'action de l'enzyme cytosolique *Δ^4-3-oxosteroid 5ß-reductase* (ou *Aldo-Keto Reductase 1 D1*, Akr1d1). Cette étape implique qu'ils soient transportés d'un environnement hydrophobe (membranes microsomales où sont localisées les enzymes 3β-Hsd et Cyp8b1) à un environnement hydrophile (cytosol). Les mécanismes permettant le trafic des intermédiaires d'un compartiment à un autre restent à ce jour méconnus (46).

Les dernières étapes conduisant à la synthèse des acides biliaires primaires consistent en l'oxydation et le raccourcissement progressifs de la chaîne latérale du cholestérol (46). Chez l'Homme, le CA et le CDCA sont produits en quantité équivalente. Chez la souris et l'ours, le CDCA est converti, respectivement, en acides muricholique et ursodéoxycholique (*Ursodeoxycholic Acid*, UDCA), plus hydrophiles et donc moins toxiques que le CDCA (52).

3.1.b. Conjugaison

Avant d'être sécrétés dans les voies biliaires, les acides biliaires primaires sont conjugués à des acides aminés taurine ou glycine, au niveau de leur chaîne latérale, donnant ainsi naissance aux sels biliaires tauro- et glyco-conjugués. Cette réaction est catalysée par les enzymes *Bile Acid-CoA : amino acid N-acyltransferase* (Baat) (53) et *Bile Acid Coenzyme A Synthase* (Bacs), particulièrement efficaces puisque près de 98% des acides biliaires excrétés du foie sont conjugués. Le ratio entre glyco- et tauro-conjugués (3 pour 1 en condition normale chez l'Homme) dépend de la disponibilité de chacun de ces deux acides aminés. La conjugaison permet de diminuer la toxicité des acides biliaires. Elle est par ailleurs nécessaire à leur solubilisation dans la bile, et leur confère leur propriété amphipathique (ils possèdent à la fois un groupement hydrophile et un groupement hydrophobe) à l'origine de leur propriété émulsifiante permettant leur action physiologique dans l'intestin.

3.1.c. Bio-transformation intestinale

Au cours de leur transit intestinal, les acides biliaires primaires vont subir une série de modifications structurales par la flore bactérienne, consistant majoritairement en leur déconjugaison et leur 7α-déhydroxylation (54). La déconjugaison est catalysée par des *Bile Salt Hydrolases* (Bsh) appartenant à la famille des *choloyl hydrolases* (Ec 3.5.1.24), et exprimées par différentes espèces de bactéries (54). Une partie de ces acides biliaires déconjugués seront absorbés par les entérocytes et redirigés vers le foie où ils seront reconjugués. La déconjugaison est un pré-requis pour la 7α-déhydroxylation, restreinte aux acides biliaires libres (55)-(56). Cette étape enzymatique donnera naissance aux acides

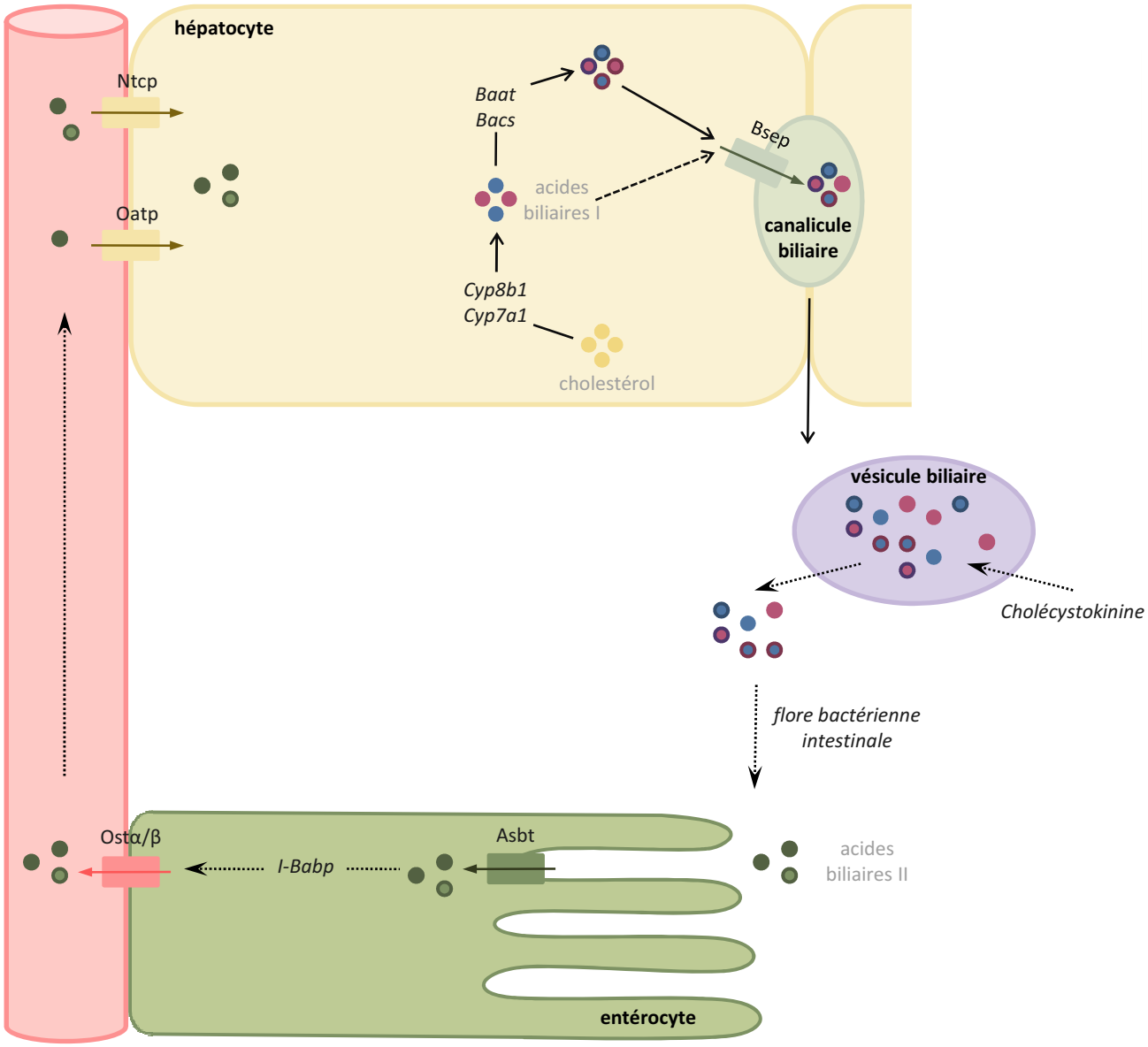


Figure 6: Représentation schématique du cycle entéro-hépatique des acides biliaires. Les acides biliaires primaires néo-synthétisés dans le foie sont pour la majorité conjugués aux acides aminés taurine ou glycine par les enzymes Baat et Bacs. Ils sont activement sécrétés dans le canalicule biliaire par le transporteur Bsep, et seront stockés dans la vésicule biliaire jusqu'au moment du repas. Ils sont alors déversés dans la lumière intestinale où ils seront convertis en acides biliaires secondaires. L'ensemble du pool d'acides biliaires permet la digestion et la solubilisation des graisses alimentaires. La majorité des acides biliaires est réabsorbée par les entérocytes et transportés par la veine porte jusqu'au foie. Captés au pôle basal des hépatocytes par les transporteurs Ntcp et Oatp, ils participeront à un nouveau cycle de digestion. L'excès d'acides biliaires est éliminé dans les fécès.

Cyp7a1 : cholesterol 7α-hydroxylase ; Cyp8b1 : sterol 12α-hydroxylase ; Baat : Bile Acid-CoA : amino acid N-acyltransferase ; Bacs : Bile Acid Coenzyme A Synthase ; Bsep : Bile Salt Export Pump ; Asbt : Apical Sodium-dependant Bile acid Transporter ; I-Babp : Ileal Bile Acid Binding Protein ; Ostα/β : Organic Solute Transporter α/β ; Oatp : Organic Anion Transporting Polypeptide ; Ntcp : Na+-Taurocholate Cotransport Polypeptide.

biliaires secondaires déoxycholique (3α,12α-dihydroxy-cholanic acid) et lithocholique (3α-hydroxy-cholanic acid) à partir respectivement des acides biliaires primaires cholique (3α,7α,12α-trihydroxy-cholanic acid) et chénodéoxycholique (3α,7α-dihydroxy-cholanic acid)) (47) (**Figure 5**). La déconjugaison et la 7α-déhydroxylation des acides biliaires augmentent leur hydrophobicité et leur pKa permettant leur absorption passive au travers de l'épithélium intestinal. Cependant, l'augmentation de leur hydrophobicité est associée à une augmentation de leur toxicité (54). Des concentrations excessives en acides biliaires secondaires dans les fèces, le sang et la bile seraient ainsi impliquées dans l'apparition de calculs biliaires et du cancer du colon (57).

3.1.d. Cycle entéro-hépatique

Les acides biliaires néo-synthétisés dans le foie sont activement sécrétés dans le canalicule biliaire grâce au transporteur membranaire *Bile Salt Export Pump* (Bsep, Abcb11), membre de la super-famille des transporteurs *ATP Binding Cassette* (Abc) (**Figure 6**). Ils sont ensuite stockés dans la vésicule biliaire jusqu'au moment du repas. Les contractions de la vésicule, induites par la cholécystokinine sécrétée par les cellules neuro-endocrines de l'intestin en présence des graisses et des protéines alimentaires, vont alors permettre de déverser les acides biliaires dans la lumière intestinale où ils vont agir comme des détergents pour aider à l'absorption des graisses et des vitamines liposolubles. Ils sont ensuite absorbés de façon active au niveau de la membrane apicale des cellules épithéliales de l'iléon terminal par l'*Apical Sodium-dependant Bile acid Transporter* (Asbt). Ils sont alors pris en charge par l'*Ileal Bile Acid Binding Protein* (I-Babp), pour être transportés de la surface apicale vers la membrane baso-latérale des entérocytes où ils sont exportés dans la veine porte par l'hétérodimère *Organic Solute Transporter* Ostα/Ostβ pour être redirigés vers le foie. Ils sont alors captés par les hépatocytes par le *Na^+-Taurocholate Cotransport Polypeptide* (Ntcp) ou par des systèmes indépendants du sodium représentés par les membres de la super-famille des *Organic Anion Transporting Polypetides* (Oatp). Après leur captage, et si nécessaire leur reconjugaison, les acides biliaires sont transportés vers le pôle canaliculaire des hépatocytes pour être sécrétés avec les acides biliaires néosynthétisés dans la bile, complétant ainsi leur cycle entéro-hépatique. 95% des acides biliaires sont réabsorbés au niveau intestinal, les 5% restants sont éliminés dans les fécès ; cette perte sera compensée par la néosynthèse hépatique.

3.1.e. Ligands de Fxrα

Les acides biliaires et leurs formes conjuguées sont donc les ligands naturels du récepteur nucléaire Fxrα (43)-(44)-(45). Ils n'ont cependant pas tous la même potentialité d'activation de Fxrα, et peuvent ainsi être classés par ordre d'affinité décroissante : CDCA > LCA = DCA > CA. Les formes

glyco- et tauro-conjuguées du CDCA, LCA et DCA sont des agonistes plus puissants que leur forme native respective. Les acides ursodéoxycholique et muricholique sont quant-à eux inactifs (23).

Il est important de noter que les acides biliaires ne sont pas des ligands spécifiques de Fxrα. Ils agissent également *via* d'autres récepteurs : le récepteur membranaire *G Protein-coupled Bile Acid Receptor 1* (Gpbar1 ou Tgr5) (58), les récepteurs nucléaires *Pregnane X Receptor* (Pxr, Nr1i2) (59)-(60), *Vitamin D3 Receptor* (Vdr, Nr1i1) (61)-(62) activables spécifiquement par le LCA, et le récepteur *Constitutive Androstan Receptor* (Car, Nr1i3) dont l'activité transcriptionnelle est réprimée par plusieurs acides biliaires, le plus efficace étant une forme conjuguée du DCA (63).

3.2. Autres ligands naturels

La guggulstérone, extraite de la résine de *Commiphora mukul*, et le stigmastérol, un phytostérol présent dans de nombreuses huiles alimentaires (soja, colza...), sont des antagonistes naturels de FXRα (64)-(65)-(66)-(67). Le cafestol, un diterpène retrouvé dans les grains de café, est quand à lui un agoniste de FXRα (68).

3.3. Ligands synthétiques

Il existe ainsi de nombreux ligands naturels pour Fxrα, mais ceux-ci ont une sélectivité modérée pour ce dernier. Ceci a conduit à l'élaboration de molécules synthétiques, plus affines et plus sélectives pour Fxrα, grâce à la modification de la structure de ligands déjà existants. Ainsi, le *6α-ethyl-chenodeoxycholic acid* (6-ECDCA aussi appelé INT-747) synthétisé à partir du CDCA est un agoniste de Fxrα (69). Le GW4064 (70), actif aussi bien *in vitro* qu'*in vivo*, est largement utilisé comme un agoniste puissant et sélectif de FXRα, et est considéré comme la référence dans ce domaine.

C. Rôles physiologiques de Fxrα

L'étude de modèles animaux invalidés pour Fxrα, et l'utilisation de ligands spécifiques de ce récepteur, ont permis de mettre en évidence son rôle clé dans diverses fonctions physiologiques, notamment dans le contrôle des métabolismes lipidique et glucidique. Les souris dont le gène codant Fxrα a été invalidé ($Fxr\alpha^{-/-}$) présentent en effet une dérégulation de l'homéostasie des acides biliaires, une élévation des concentrations plasmatique et intra-hépatique de cholestérol et de triglycérides (71), ainsi qu'une intolérance au glucose couplée à une résistance à l'insuline (72)-(32)-(73). Certains mécanismes moléculaires impliqués ont été identifiés.

1. Fxrα et acides biliaires

De part leur action détergente, une accumulation excessive d'acides biliaires est délétère pour les cellules et l'organisme. Fxrα intervient à plusieurs niveaux du cycle entéro-hépatique des acides

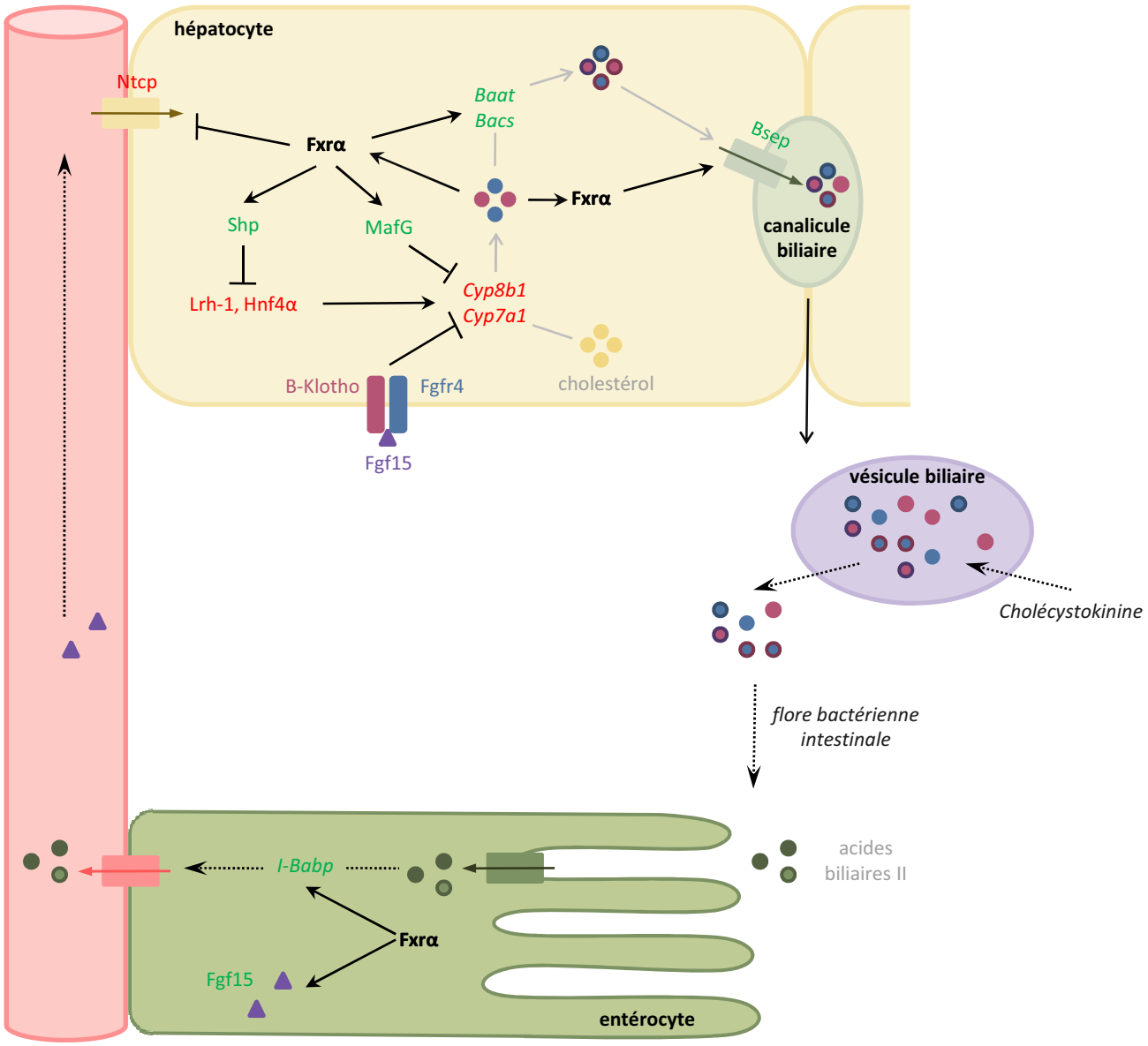


Figure 7: Fxrα contrôle l'homéostasie des acides biliaires. Fxrα intervient à plusieurs niveaux du cycle entéro-hépatique des acides biliaires afin de maintenir leur homéostasie et limiter leur accumulation délétère pour les cellules. Au niveau hépatique, il réprime l'expression des deux enzymes clés de cette voie de biosynthèse, Cyp7a1 et Cyp8b1, par des mécanismes moléculaires impliquant d'une part les récepteurs nucléaires Shp, Lrh-1 et Hnf4α, et d'autre part le facteur de transcription MafG. Dans les entérocytes, l'activation de Fxrα par les acides biliaires induit la synthèse et la libération de l'hormone Fgf15. Conduite jusqu'au foie, elle active les récepteurs Fgfr4 et β-Klotho ce qui conduit à la répression de l'expression de Cyp7a1. Parallèlement, Fxrα protège le foie de l'accumulation toxique des acides biliaires en favorisant leur efflux et limitant leur retour hépatique grâce respectivement à l'induction et à la répression de l'expression des transporteurs hépatiques Bsep et Ntcp. Il participe à la détoxication des acides biliaires en favorisant leur conjugaison *via* l'augmentation de la transcription des enzymes Bacs et Baat. Enfin, il protège les entérocytes de la toxicité des acides biliaires en activant l'expression I-Babp.

Fxrα : Farnesoid X Receptor alpha ; Cyp7a1 : cholesterol 7α-hydroxylase ; Cyp8b1 : sterol 12α-hydroxylase ; Baat : Bile Acid-CoA : amino acid N-acyltransferase ; Bacs : Bile Acid Coenzyme A Synthase ; Bsep : Bile Salt Export Pump ; I-Babp : Ileal Bile Acid Binding Protein ; Ntcp : Na+-Taurocholate Cotransport Polypeptide ; Shp : Small Heterodimer Partner ; Lrh-1 : Liver Receptor Homolog-1 ; Hnf4α : Hepatocyte Nuclear Factor 4α ; Fgfr4 : Fibroblast Growth Factor Receptor 4.

biliaires afin de maintenir leur homéostasie, et de limiter leur accumulation dans les hépatocytes : il réprime la néo-synthèse hépatique des acides biliaires, favorise leur excrétion biliaire et leur détoxication, et limite leur retour hépatique (**Figure 7**).

1.1.Contrôle de la synthèse des acides biliaires

1.1.a. Au niveau hépatique

Fxrα limite la synthèse des acides biliaires en réprimant l'expression de deux enzymes clés de cette voie de biosynthèse : Cyp7a1 et Cyp8b1 (30)-(74)-(75). Les mécanismes moléculaires mis en jeu impliquent principalement trois autres membres de la famille des récepteurs nucléaires : Shp, Lrh-1 et *Hepatocyte Nuclear Factor 4α* (Hnf4α). Shp est un récepteur nucléaire particulier, dépourvu de domaine de liaison à l'ADN, capable d'interagir avec d'autres récepteurs nucléaires afin d'inhiber leur activité transcriptionnelle (22). Lrh-1 se lie sous forme de monomère sur un élément de réponse au niveau du promoteur de Cyp7a1 afin d'initier sa transcription (76)-(77)-(78). Hnf4α est quant-à lui un régulateur clé de l'expression du gène codant l'enzyme Cyp8b1 (75). Fxrα en activant la transcription de Shp, interfère avec l'action de Lrh-1 et de Hnf4α sur le promoteur des gènes codant respectivement les enzymes Cyp7a1 et Cyp8b1, réprimant ainsi leur transcription.

Par ailleurs, le facteur de transcription *v-maf musculoapo-neurotic fibrosarcoma oncogene homolog G (avian)* (MafG), gène cible de Fxrα, a été récemment impliqué dans le contrôle de l'expression du gène codant Cyp8b1 par Fxrα (79). Ainsi, la surexpression de MafG entraîne-t-elle *in vivo* la répression de Cyp8b1, associée à une modification de la composition du pool d'acides biliaires, favorisant la synthèse de l'acide muricholique au détriment de l'acide cholique. L'analyse des animaux hétérozygotes $MafG^{+/-}$ a révélé une dérepression de l'expression de Cyp8b1 avec des changements concordants dans la composition du pool d'acides biliaires.

1.1.b. Au niveau intestinal

La régulation intra-hépatique est complétée, au niveau intestinal, par l'action de Fxrα sur le facteur de croissance Fgf15 (souris) / FGF19 (Homme) (80)-(81). Synthétisé dans les cellules épithéliales iléales, il agit de façon endocrine sur les hépatocytes *via* son récepteur *Fibroblast Growth Factor Receptor 4* (Fgfr4) afin de réprimer l'expression de Cyp7a1. L'activation de Fgfr4 nécessite la présence d'un partenaire membranaire, β-Klotho, et enclenche les voies de signalisation intra-cytoplasmique *Extracellular signal-Related Kinase* (Erk) et *Jun N-terminal Kinase* (Jnk) (82)-(83). L'importance de cette cascade de signalisation est soulignée par une récente étude, montrant que la réexpression intestinale de Fxrα chez des souris $Fxr\alpha^{-/-}$ suffit à restaurer le rétrocontrôle de la synthèse hépatique des acides biliaires (84).

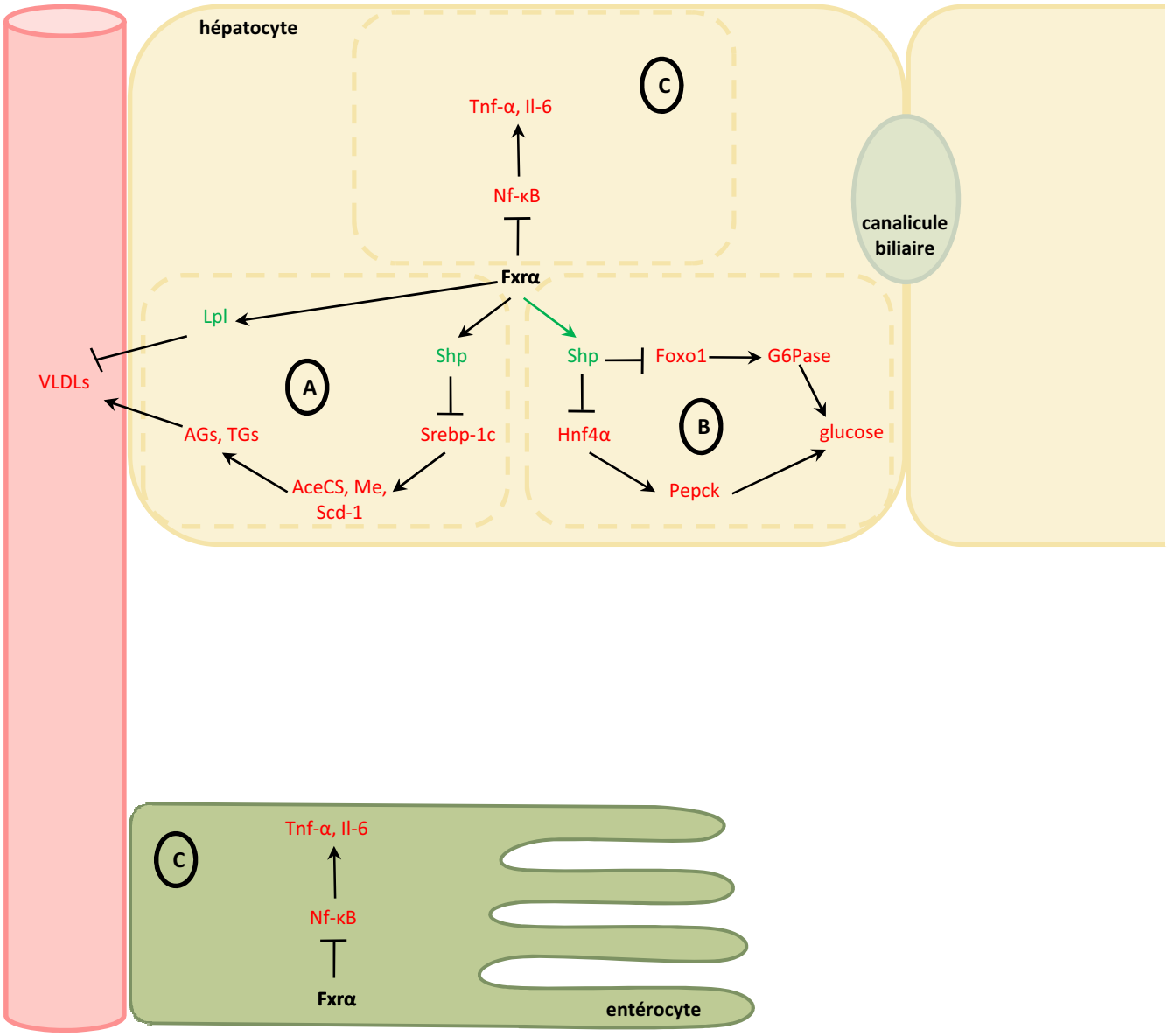


Figure 8: Rôles physiologiques de Fxrα. A. Fxrα limite la synthèse des triglycérides. Fxrα, via la régulation de la transcription de Shp, inhibe l'expression du facteur de transcription Srebp-1c et de ses gènes cibles AceCs, Me et Scd-1 impliqués dans la biosynthèse hépatique des acides gras et des triglycérides. Par ailleurs, il favorise la clairance des lipoprotéines VLDL en favorisant l'activité de la Lpl. **B.** Fxrα réprime la néoglucogenèse. Fxrα exerce un rôle hypoglycémiant en activant l'expression de Shp, ce qui conduit à l'inhibition de l'activité transactivatrice des facteurs de transcription Hnf4α et Foxo1 sur le promoteur des enzymes hyperglycémiantes Pepck et G6Pase respectivement. **C.** Fxrα joue un rôle anti-inflammatoire. Dans le foie et les entérocytes, Fxrα réprime l'activité de Nf-κB sur le promoteur des cytokines inflammatoires Tnf-α et Il-6.

Fxrα : Farnesoid X Receptor alpha ; Shp : Small Heterodimer Partner ; Srebp-1c : Sterol Regulatory Element Binding Protein-1c ; AceCS : Acetyl-CoA Synthetase ; ME : Malic Enzyme ; Scd-1 : Stearoyl-CoA Desaturase-1 ; Lpl : Lipoprotein Lipase ; VLDL : Very Low-Density Lipoprotein ; Hnf4α : Hepatocyte Nuclear Factor 4α ; Pepck : Phosphoenolpyruvate Carboxykinase ; Foxo1 : Forkhead box protein O1 ; G6Pase : Glucose-6-Phosphatase ; Tnf-α : Tumor Necrosis Factor-α ; Il-6 : Interleukine-6.

1.2. Contrôle du transport des acides biliaires

Fxrα protège les hépatocytes et les entérocytes de l'effet toxique d'une accumulation excessive d'acides biliaires. Il favorise l'excrétion des acides biliaires dans les voies biliaires en activant la transcription du transporteur Bsep (85). Dans l'intestin, il active l'expression de la protéine I-Babp qui facilitera le transport des acides biliaires à travers les entérocytes (86)-(87). Enfin, il diminue le retour hépatique des acides biliaires en réprimant, *via* Shp, l'expression du transporteur Ntcp (88).

1.3. Rôle dans la détoxication des acides biliaires

Fxrα participe à la détoxication des acides biliaires en favorisant leur conjugaison *via* l'augmentation de la transcription des enzymes Bacs et Baat par un mécanisme direct (89). Il a également pour cible les enzymes *dehydroepiandrosterone-sulfotransferase* (Sult2a1) (90), *Uridine Glucuronosyltransferase* (Ugt2b4) (91) et Cyp3a4 (92), impliquées respectivement dans la sulfatation (93), la glucuronidation (94)-(95) et l'oxydation (96) des acides biliaires permettant de diminuer leur toxicité.

2. Fxrα et triglycérides

Les acides biliaires sont connus depuis longtemps pour affecter l'homéostasie des triglycérides. En effet, l'utilisation de séquestrants d'acides biliaires chez des patients hypercholestérolémiques (97)-(98)-(99)-(100), ou la réalisation d'une résection iléale (101), aboutissant toutes deux à l'interruption de la circulation entéro-hépatique des acides biliaires, se traduisent par une élévation de la concentration plasmatique de triglycérides. A l'inverse, ce paramètre est diminué en réponse à l'administration d'acides biliaires pour le traitement de patients atteints d'hypertriglycéridémie (97)-(102)-(103), ou de calculs biliaires (104).

En 2004, les travaux de Watanabe *et al* (31) ont permis d'identifier Fxrα comme étant le lien moléculaire entre les acides biliaires et les triglycérides. Fxrα, *via* la régulation de la transcription de Shp, inhibe l'expression du facteur de transcription *Sterol Regulatory Element Binding Protein-1c* (Srebp-1c) et de ses gènes cibles *Acetyl-CoA Synthetase* (AceCs), *Malic Enzyme* (Me) et *Stearoyl-CoA Desaturase-1* (Scd-1) impliqués dans la biosynthèse des acides gras et des triglycérides (**Figure 8**). Ces résultats sont cohérents avec le profil lipidique des souris Fxrα$^{-/-}$, qui révèle une augmentation des concentrations intra-hépatiques et plasmatiques de triglycérides, ainsi que des lipoprotéines de très faible densité (*Very Low-Density Lipoprotein*, VLDL) (71). Outre la régulation de leur synthèse, Fxrα contribue à limiter l'accumulation des triglycérides circulants en favorisant également leur élimination. La concentration plasmatique de triglycérides est en effet le résultat de la balance entre leur synthèse hépatique, et la clairance des lipoprotéines riches en triglycérides telles que les VLDL. Cette dernière est assurée par des lipases, notamment par la *Lipoprotein Lipase* (Lpl), dont l'activité

est modulée par plusieurs co-facteurs : les *Apolipoprotein C-II* (ApoC-II) et A-V (ApoA-V) sont des activateurs de la Lpl, tandis que l'apoC-III est un inhibiteur de son activité. Les ApoC-II et apoA-V sont activées par Fxrα (105)-(106)-(107), tandis que l'activation de Fxrα conduit à la répression de l'expression de l'apoC-III chez la souris et dans des hépatocytes de culture primaire humaine (108). De cette manière, Fxrα favorise la clairance des VLDL par l'intermédiaire de la Lpl.

3. Fxrα et glucose

Le diabète de type 2 est une pathologie qui s'accompagne souvent d'une hypertriglycéridémie, et constitue un facteur de risque pour le développement de troubles de la vésicule biliaire (109)-(110). Récemment, un lien a été établi entre ces deux complications, l'hypertriglycéridémie favorisant la formation de calculs vésiculaires (111). De plus, la composition de la bile est altérée chez certains patients diabétiques (112)-(113). Fxrα étant connu pour contrôler l'homéostasie des acides biliaires et des triglycérides, des études ont été menées afin de déterminer l'existence d'un lien potentiel entre Fxrα et le métabolisme glucidique. Il a ainsi été montré que l'expression de Fxrα est modulée par les variations glycémiques. Son expression hépatique est en effet diminuée chez un modèle de rat diabétique (114). Ces mêmes auteurs ont montré que l'accumulation des messagers de Fxrα est augmentée en réponse au glucose, et diminuée par l'insuline, dans une culture d'hépatocytes primaires. Ainsi, l'altération de l'expression de Fxrα en réponse à la modification de l'homéostasie glucidique pourrait-elle participer au développement de l'hypertriglycéridémie et à la dérégulation du métabolisme des acides biliaires chez les patients atteints de diabète ou de résistance à l'insuline.

Ce lien établi entre Fxrα et glucose est en fait bi-directionnel, puisque Fxrα contrôle en retour le métabolisme glucidique. Plusieurs études suggéraient un impact des acides biliaires sur l'expression de gènes impliqués dans la néoglucogenèse (115)-(116)-(117). Ma *et al* (2006) (32) ont montré que les acides biliaires exercent leur action *via* la voie de signalisation moléculaire Fxrα/Shp. Un régime enrichi en CA aboutit en effet à la répression de l'expression de gènes néoglucogéniques tels que *Phosphoenolpyruvate Carboxykinase* (Pepck) et *Glucose-6-Phosphatase* (G-6-Pase), et à la diminution des concentrations de glucose chez des souris sauvages. Ces effets sont complètement abolis chez les souris invalidées pour Fxrα ou Shp. Shp agit en inhibant l'activité transactivatrice des facteurs de transcription Hnf4α et *Forkhead box protein O1* (Foxo1) sur le promoteur des enzymes hyperglycémiantes (Pepck et G-6-Pase respectivement) (116) (**Figure 8**). De plus, l'activation de Fxrα par le GW4064 chez des souris diabétiques db/db induit une diminution de l'expression de la Pepck et de la G-6-Pase, associée à une diminution de la glycémie plasmatique. Enfin, la surexpression hépatique d'une forme constitutivement active de Fxrα conduit à une diminution des niveaux plasmatiques de glucose chez des souris non diabétiques (73).

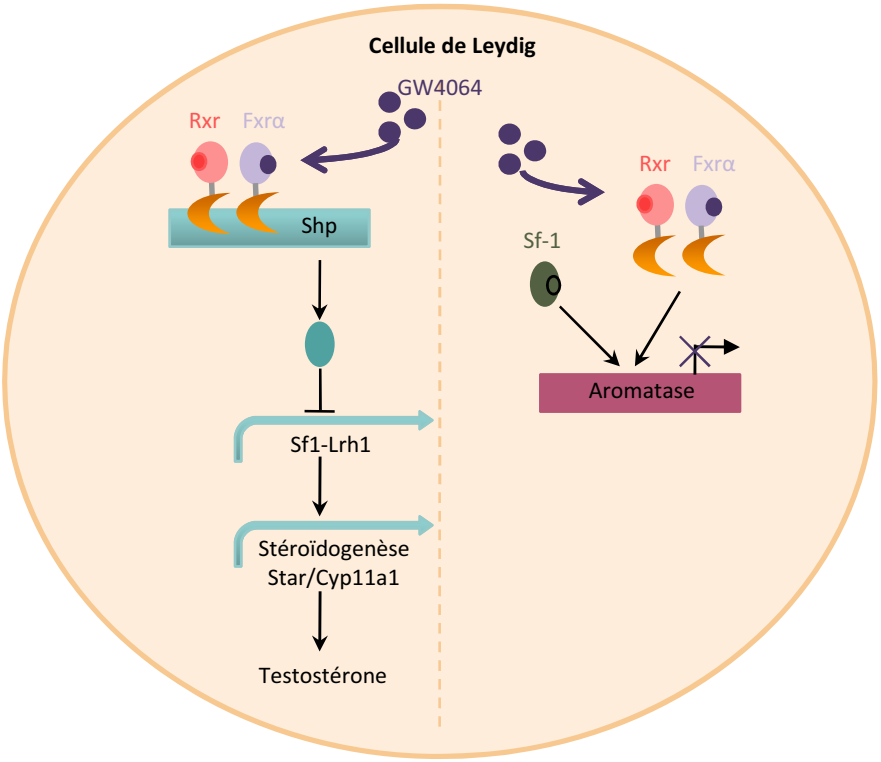


Figure 9: Fxrα contrôle la stéroïdogenèse dans les cellules de Leydig. L'activation *in vivo* de Fxrα conduit à court terme à la répression de la stéroïdogenèse, se traduisant par une diminution de l'accumulation des transcrits de Star, Cyp11a1 et 3βHsd, par un mécanisme dépendant des récepteurs nucléaires Shp, Sf-1 et Lrh-1. Dans une lignée de cellules de Leydig tumorales, Fxrα entre en compétition avec Sf-1 sur le promoteur de l'aromatase, ce qui inhibe son expression.

Fxrα : Farnesoid X Receptor alpha ; Shp : Small Heterodimer Partner ; Sf-1 : Steroidogenic Factor-1 ; Lrh-1 : Liver Receptor Homolog-1 ; Star : Steroidogenic Acute Regulatory protein ; Cyp11a1 : Cytochrome P450 Side-Chain Cleavage ; 3ϐHsd : 3ϐ Hydroxysteroid Dehydrogenase.

4. Fxrα et stéroïdes

4.1. glucocorticoïdes

Fxrα est fortement exprimé dans les cellules corticales surrénaliennes de la zone fasciculée (118)-(119), siège de la synthèse des glucocorticoïdes. Le traitement de souris avec le ligand synthétique de Fxrα, le GW4064, conduit à une élévation des concentrations plasmatiques de la corticostérone (120). Cette augmentation de la stéroïdogenèse surrénalienne est liée à un captage plus important du substrat de la stéroïdogenèse, le cholestérol. Fxrα induit en effet l'expression du transporteur *Scavenger Receptor class B, member 1* (Sr-b1), qui assure le captage des esters de cholestérol à partir des lipoprotéines de haute densité (*High-Density Lipoprotein*, HDL). Une action répressive de Fxrα sur l'enzyme *UDP-Glucuronosyltransferase 2 family, polypeptide B7* (UGT2B7), responsable du catabolisme des hormones stéroïdes surrénaliennes, a par ailleurs été mise en évidence dans les cellules tumorales intestinales humaines Caco-2 (121) : cette action périphérique pourrait contribuer à augmenter davantage les concentrations des glucocorticoïdes.

4.2. hormones sexuelles

Deux études indépendantes ont identifié Fxrα dans les cellules de Leydig chez la souris adulte (33)-(122). L'analyse des animaux invalidés pour Fxrα a révélé des niveaux plasmatiques de testostérone équivalents à ceux des souris sauvages, ce qui est corrélé au niveau moléculaire par une accumulation basale normale des transcrits de gènes codant les enzymes de la stéroïdogenèse (*Steroidogenic Acute Regulatory protein* (star) , *Cytochrome P450 Side-Chain Cleavage* (cyp11a1) , *3-β Hydroxysteroid Dehydrogenase* (3βhsd)). En revanche, l'injection de l'agoniste synthétique GW4064 à des souris adultes conduit à la répression de la stéroïdogenèse, se traduisant par une diminution de l'accumulation des transcrits de star, cyp11a1 et 3βhsd, et par un effondrement des concentrations plasmatique et intra-testiculaire de testostérone. Cet effet répresseur est pour partie indépendant de l'axe hypothalamo-hypophysaire, et fait intervenir le récepteur nucléaire Shp (**Figure 9**). Celui-ci agit d'une part en inhibant l'expression de Sf-1 et Lrh-1, deux récepteurs nucléaires connus pour contrôler la transcription de plusieurs gènes impliqués dans la stéroïdogenèse (123)-(124)-(125) ; d'autre part Shp interagit avec Lrh-1 inhibant de ce fait son activité transactivatrice (33).

Il est intéressant de noter que, outre les acides biliaires, Fxrα peut également être activé par l'androstérone, un métabolite de la testostérone (126). Fxrα pourrait ainsi participer à un rétrocontrôle local de la synthèse des androgènes.

Par ailleurs, l'activation de Fxrα par l'utilisation d'un ligand naturel (le CDCA), ou synthétique (le GW4064) conduit à la répression de l'expression et de l'activité enzymatique de l'aromatase dans

une lignée tumorale de Leydig de rat. Le mécanisme est indépendant de Shp, et fait intervenir un mécanisme de compétition entre Fxrα et Sf-1 pour la fixation de ces récepteurs nucléaires sur le promoteur de l'aromatase (38) (**Figure 9**).

5. Fxrα et cancer

Les souris invalidées pour Fxrα développent spontanément à partir de l'âge de 1 an des lésions hépatiques cancéreuses (127)-(128). Par ailleurs, la perte de Fxrα favorise la prolifération cellulaire, l'inflammation et la tumorigenèse dans l'intestin (129).

Le cancer et l'inflammation chronique sont étroitement liés. Le carcinome hépatocellulaire est ainsi une complication fréquente de maladies inflammatoires chroniques telles que la stéatose hépatique, caractérisée par un excès de graisses dans le cytoplasme des hépatocytes, et favorisée notamment par le diabète. De part sa fonction régulatrice des métabolismes lipidique et glucidique, Fxrα permettrait de prévenir les troubles métaboliques hépatiques et leur évolution potentielle en carcinome hépatocellulaire.

Par ailleurs, une augmentation de l'expression de facteurs pro-inflammatoires tels que l'interféron γ, le *Tumor Necrosis Factor-α* (Tnf-α), et l'*Interleukine-6*, a été observée dans le foie et le colon des souris invalidées pour Fxrα par rapport aux souris sauvages (128)-(129). L'inflammation associée à certaines pathologies chroniques hépatiques et intestinales serait attribuée principalement à l'activation du facteur de transcription NF-κB (130). L'activation de Fxrα par le GW4064 inhibe l'activité transcriptionnelle de NF-κB dans les cellules hépatiques et intestinales *in vitro* (131)-(132). Fxrα, *via* son action anti-inflammatoire, pourrait donc participer à la prévention du développement tumoral.

Les acides biliaires semblent être impliqués dans l'apparition de tumeurs hépatiques et intestinales. Les enfants atteints de cholestase intra-hépatique progressive familiale de type 2 (*progressive familial intrahepatic cholestasis type 2* (PFIC type 2)), caractérisée par une élévation des concentrations plasmatique et intra-hépatique d'acides biliaires, sont en effet prédisposés au carcinome hépatocellulaire (133). De plus, l'administration d'acides biliaires exogènes à des rats (134)-(135) ou des souris (128) favorise la tumorigenèse hépatique. La consommation d'une alimentation riche en graisse est un facteur de risque bien connu pour le développement d'un cancer du colon, et s'accompagne chez ces patients d'une augmentation de la concentration fécale d'acides biliaires (136). L'exposition des cellules hépatiques et épithéliales colorectales à de fortes concentrations d'acides biliaires pourrait ainsi participer au processus tumoral. Fxrα, en contrôlant

l'homéostasie des acides biliaires, pourrait exercer un rôle protecteur vis-à-vis de la tumorigenèse hépatique et intestinale.

6. polymorphismes du gène codant Fxrα

Etant donné le rôle central joué par Fxrα dans le maintien de l'homéostasie du cholestérol, des acides biliaires, des triglycérides ou encore du glucose, il est concevable que l'existence de polymorphismes génétiques de ce récepteur nucléaire, affectant son expression ou son activité, pourrait prédisposer l'organisme au développement de pathologies associées à ces métabolites (calculs biliaires, cholestase, diabète...). En 2007, une étude à grande échelle s'est intéressée à la recherche de polymorphismes de FXRα dans une cohorte de sujets rassemblant des européens, des africains, des chinois et des hispano-américains (137). Plusieurs *Single Nucleotide Polymorphisms* (SNPs) ont été identifiés, parmi lesquels le SNP G-1T retrouvé à une fréquence de 2,5 à 12,1% en fonction des groupes ethniques, donnant naissance à un variant de FXRα ayant une activité transcriptionnelle réduite *in vitro*. De plus, ce polymorphisme est associé *in vivo* à une diminution de l'expression hépatique des gènes cibles de FXRα, SHP et OATP1B3. Ce SNP a été identifié la même année chez des patientes ayant développé une cholestase gravidique (*Intrahepatic Cholestasis of Pregnancy*, ICP) (138). Il pourrait donc, avec les autres SNPs identifiés dans cette deuxième étude, prédisposer à l'ICP, et à tout autre désordre d'ordre cholestatique ou dyslipidémique. A ce jour, ce SNP est l'un des rares pour FXRα à avoir été identifié comme ayant une conséquence fonctionnelle, et à avoir été associé au développement d'une pathologie.

7. Régulation de l'expression et de l'activité de Fxrα

Fxrα est soumis à des modifications post-traductionnelles affectant son activité, catalysées notamment par les co-facteurs qui lui sont associés. Fxrα peut ainsi être acétylé (139), phosphorylé (140)-(141), ubiquitinylé (142), sumoylé (143) et méthylé (144). Fxrα est la cible des acteurs p300 et Sirtuin 1 (Sirt1), catalysant respectivement son acétylation et sa déacétylation. L'acétylation de Fxrα augmente sa stabilité, mais inhibe son hétérodimérisation avec Rxr, sa fixation à l'ADN et son activité trans-activatrice sur ses gènes cibles. De façon intéressante, l'acétylation de Fxrα a été retrouvée constitutivement élevée dans un modèle murin atteint d'un syndrome métabolique. L'utilisation de molécules thérapeutiques ciblant p300 ou Sirt1 afin de moduler le statut d'acétylation de Fxrα pourrait donc se révéler prometteuse dans le cadre du traitement de cette pathologie (139). Deux équipes indépendantes ont démontré que Fxrα peut être phosphorylé par la *Protein Kinase C* (PkC) (140)-(141). La phosphorylation de Fxrα favorise le recrutement du co-activateur transcriptionnel *peroxisomal Proliferator-activated receptor Gamma Coactivator-1alpha* (Pgc-1α), et stimule son activité trans-activatrice. Fxrα peut être ubiquitinylé *in vitro*, et Kemper *et al* (2011) ont montré que

l'utilisation d'un inhibiteur du protéasome augmente de façon significative l'accumulation protéique de Fxrα, suggérant que ce dernier peut subir une dégradation protéasomale (142). Enfin, Fxrα peut être soumis à la sumoylation et à la méthylation par la méthyltransférase Set7/9, aboutissant respectivement à la diminution et à la stimulation de l'activité trans-activatrice de Fxrα sur ses gènes cibles (143)-(144).

La méthylation régule d'autre par l'expression de Fxrα au niveau transcriptionnel (145). En effet, une équipe a mis en évidence une diminution de l'expression de Fxrα, corrélée à la méthylation de son promoteur, chez des patients souffrant d'un cancer du colon.

D'autres acteurs ont été impliqués dans le contrôle transcriptionnel de l'expression de Fxrα. Celle-ci est diminuée en réponse à une réaction inflammatoire, et des études *in vitro* ont montré que cette régulation implique les cytokines Tnf et Il-1, ainsi que l'activation du facteur de transcription *Signal Transducer And Activator Of Transcription 1* (STAT1) dépendante de l'interféron gamma (146)-(147). Par ailleurs, Fxrα a été récemment identifié comme un gène cible du facteur de transcription *Caudal-related homeobox 2* (Cdx2), qui stimule son expression dans les entérocytes, ce qui en fait un candidat intéressant pour moduler l'expression de Fxrα contre la progression du cancer colorectal causée par les acides biliaires (148).

8. Fxrα : une cible thérapeutique de choix

De part son rôle clé dans le contrôle du métabolisme des acides biliaires, des triglycérides et du glucose, Fxrα constitue une cible thérapeutique particulièrement intéressante pour le traitement de troubles hépatiques, de la dyslipidémie ou encore du diabète. Plusieurs études cliniques portant sur des agonistes ou antagonistes de Fxrα ont été menées en ce sens (149). Le 6-ECDCA a fait l'objet d'un test clinique de phase 2 pour évaluer son impact sur la sensibilité à l'insuline chez des patients atteints de stéatose hépatique non alcoolique et de diabète de type 2 (150). Les résultats de cette étude sont prometteurs, puisque l'administration de cet agoniste de Fxrα a été bien tolérée, et a abouti à une amélioration de la sensibilité à l'insuline, et une diminution des marqueurs hépatiques d'inflammation. Au vu de l'action répressive exercée par Fxrα sur la stéroïdogenèse testiculaire chez la souris, la question de l'impact de tels traitements sur la physiologie testiculaire et les capacités de reproduction devrait être prise en considération.

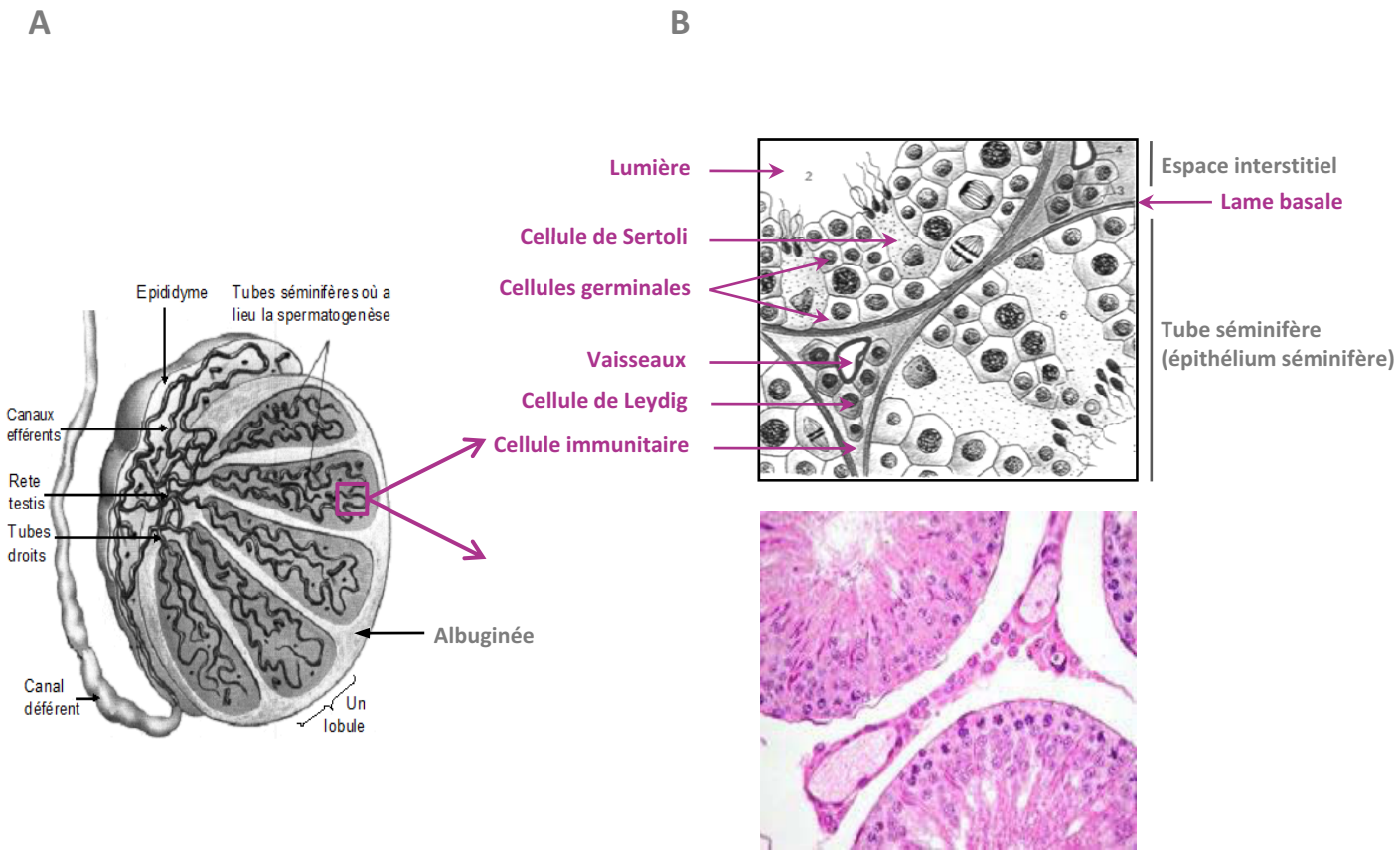


Figure 10 : Anatomie et histologie du testicule. A. Représentation schématique de la structure interne du testicule. Le testicule est recouvert d'une capsule conjonctive épaisse, l'albuginée. Celle-ci le découpe en lobules, renfermant chacun plusieurs dizaines de tubes séminifères entourés du tissu interstitiel. **B.** Représentation schématique et coupe histologique du testicule. L'épithélium séminifère est constitué des cellules de Sertoli et des différents stades de différenciation des cellules germinales. L'espace interstitiel renferme les cellules de Leydig et les cellules immunitaires, et est traversé par des vaisseaux sanguins et lymphatiques. Adapté d'après Pr. George Dolisi.

Partie 2 : La physiologie du testicule

Le testicule est une glande paire et symétrique de l'appareil reproducteur mâle, possédant une activité exocrine (la production des spermatozoïdes) et une activité endocrine (la synthèse d'hormones, en particulier des androgènes). Localisés au niveau de la partie antérieure du périnée de part et d'autre du pénis, les testicules sont suspendus par les cordons spermatiques dans une poche cutanée protectrice appelée scrotum, située à l'extérieur de la cavité abdominale. Cette localisation anatomique permet de maintenir les testicules à une température légèrement inférieure à celle du corps, condition indispensable pour le développement de spermatozoïdes viables (151).

Chaque testicule est recouvert d'une capsule conjonctive épaisse, l'albuginée. Composée de fibres de collagène et de cellules musculaires lisses, elle envoie des cloisons ou ''*septa*'' qui découpent le testicule en environ 300 lobules testiculaires. Chacun de ces lobules renferme entre 15 et 40 tubes séminifères entourés d'un tissu conjonctif lâche nommé espace interstitiel (**Figure 10**).

Les tubes séminifères sont le siège de la production des spermatozoïdes, qui sont le ''véhicule'' à travers lequel est transmise l'information génétique mâle aux générations successives. Le processus de spermatogenèse est supporté par de fortes interactions s'établissant entre les cellules germinales et les cellules somatiques de ces tubes, que sont les cellules de Sertoli et les cellules myoïdes péritubulaires. Ces deux populations cellulaires assurent l'intégrité structurale des tubes séminifères en produisant la membrane basale nécessaire au maintien de la polarité épithéliale. Les cellules de Sertoli assurent un support structural et nutritif aux cellules germinales indispensables à leur survie et à leur différenciation. Les cellules myoïdes supportent quant-à elles l'activité contractile des tubes séminifères, sécrètent des facteurs paracrines qui modulent l'activité des cellules de Sertoli, et participent au contrôle androgénique de la spermatogenèse.

Le tissu interstitiel est traversé par de nombreux vaisseaux sanguins et lymphatiques (152), qui assurent la distribution des nutriments (ions, vitamines A et E ...), hormones (*Luteinizing Hormone* (Lh), *Follicle Stimulating Hormone* (Fsh), insuline ...), et facteurs de croissance (*Epidermal Growth Factor* (Egf) ...) nécessaires aux fonctions endocrine et exocrine du testicule. Il contient par ailleurs des cellules immunitaires, en particulier des macrophages, et les cellules de Leydig. Ces dernières sont responsables de la synthèse des stéroïdes sexuels, en particulier des androgènes qui, produits en fortes quantités, sont nécessaires au bon déroulement de la spermatogenèse et au développement des caractéristiques phénotypiques mâles.

Bien que différenciés d'un point de vue histologique, l'ensemble des types cellulaires du testicule sont étroitement liés sur le plan fonctionnel, l'activité et la survie de chacun d'entre eux dépendant

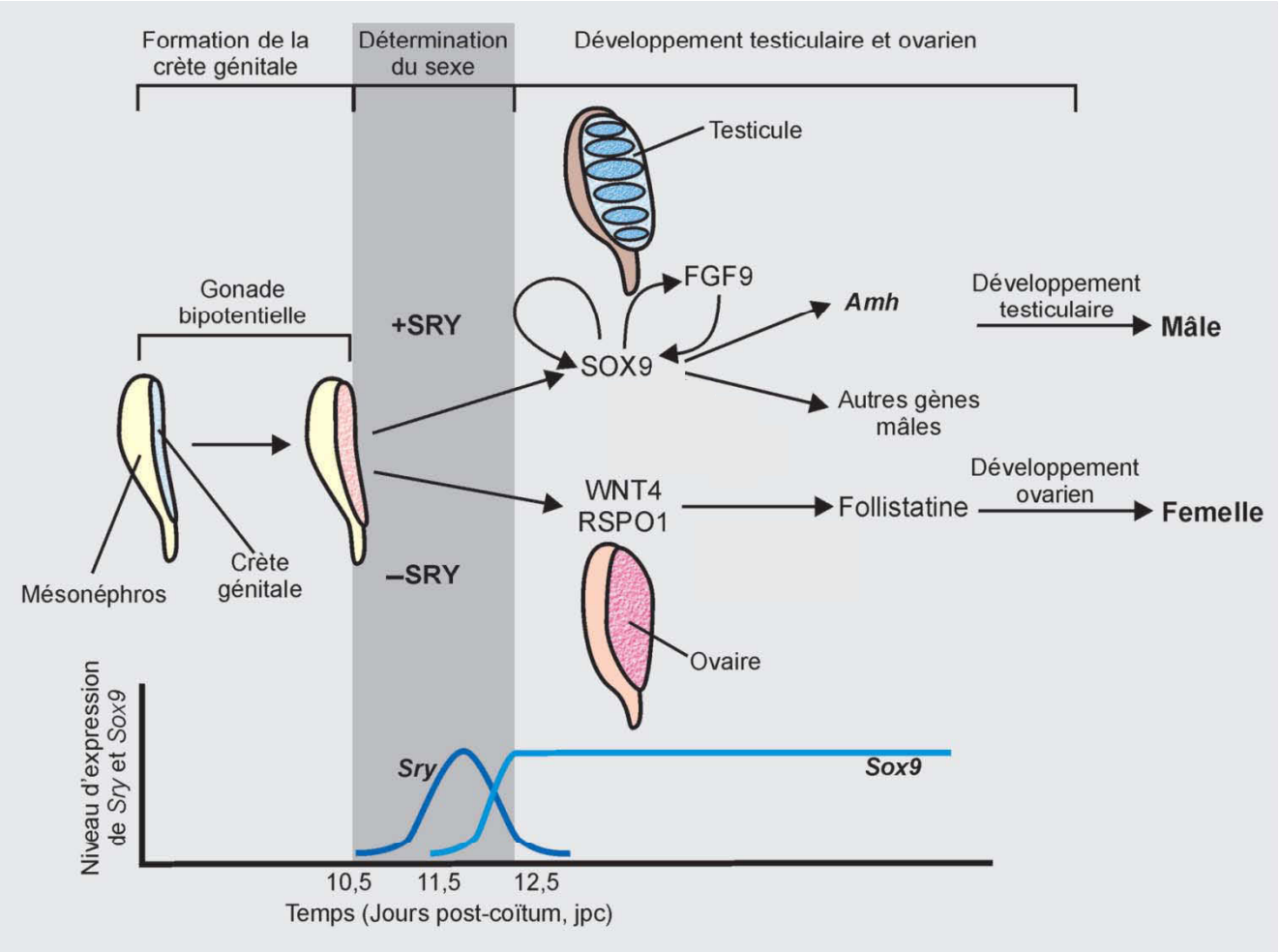


Figure 11 : La différenciation du testicule chez la souris. Les gonades se forment au cours de la première moitié de la gestation sur la face ventrale du mésonéphros, à partir d'un épaississement de l'épithélium cœlomique. Alors appelées crêtes génitales, ces ébauches gonadiques possèdent un caractère bipotentiel. La différenciation testiculaire débute à 10,5 jours *post-coïtum* avec l'expression transitoire du gène Sry dans les précurseurs des cellules de Sertoli. Sry induit l'expression de Sox9 qui sera maintenue à un niveau élevé grâce à la mise en place de plusieurs boucles de régulation. Sox9 est responsable d'une part de l'activation de l'expression de gènes impliqués dans la différenciation des types somatiques du testicule, et d'autre part de l'inhibition de l'expression de la signalisation Wnt4/RSPO1 impliquée dans le développement ovarien.
Sry : Sex determination Region on Y chromosome ; Sox9 : Sry-related HMG box 9 ; Fgf9 : Fibroblast Growth Factor 9 ; Wnt4 : Wingless-type MMTV integration site family, member 4 ; Rspo1 : R-Spondin-1 ; Amh : Anti-Müllerian Hormone. Adapté d'après Kashimada et Koopman, 2010.

des fonctions des autres types cellulaires, et des interactions inter-cellulaires. Ce dialogue fait intervenir de multiples acteurs endocriniens, autocrines, paracrines et juxtacrines. Par exemple, les cellules de Sertoli sont essentielles à l'initiation et au maintien de la spermatogenèse *via* des interactions directes avec les cellules germinales en différenciation. Le développement, l'activité et la survie des cellules de Leydig dépendent quant-à elles notamment de la présence continue des cellules de Sertoli (153)-(154).

A. Le développement du testicule chez la souris

1. Le développement fœtal

1.1. La formation de la gonade indifférenciée

Chez les mammifères, les gonades se forment pendant la vie embryonnaire, au cours de la première moitié de la gestation (au stade embryonnaire E9.5-E10 chez la souris) (**Figure 11**). Elles apparaissent initialement comme une ébauche sexuellement indifférenciée, nommée crête génitale, qui se développe de façon identique entre les deux sexes : elle prend naissance sur la face ventrale du mésonéphros par un épaississement de l'épithélium cœlomique, à partir duquel se différencieront les cellules somatiques gonadiques (155). Les cellules germinales ont quant-à elles pour origine les cellules germinales primordiales (ou *Primordial Germ Cells*, PGCs) dérivant de l'épiblaste. Celles-ci vont subir une migration extra-embryonnaire entre E10.5 et E11.5, au cours de laquelle elles s'amplifient par mitoses afin de coloniser la crête génitale. Chez le mâle, les PGCs, alors entourées par les précurseurs des cellules de Sertoli dans cette ébauche gonadique, opèrent un changement morphologique et sont dès lors appelées gonocytes (156). Les gonocytes poursuivent leur prolifération jusqu'au stade embryonnaire E14.5, où ils s'arrêtent en phase G_0/G_1 du cycle cellulaire ; ils n'entreront dans leur phase méiotique qu'au moment de la puberté.

1.2. La différenciation testiculaire

La différenciation du testicule débute au stade embryonnaire E12 avec la différenciation des cellules de Sertoli. Ces cellules s'associent les unes aux autres par des jonctions membranaires, entourant progressivement les gonocytes et donnant ainsi naissance aux cordons séminifères. Entre ces cordons, certaines cellules du mésenchyme se différencient vers E12.5 en cellules de Leydig fœtales. Le testicule fœtal sécrète très précocement deux types d'hormones : l'hormone anti müllérienne (*Anti-Müllerian Hormone*, Amh) par les cellules de Sertoli et la testostérone par les cellules de Leydig, responsables respectivement de la régression des canaux de Müller et de la masculinisation du tractus génital (157). Les cellules de Leydig fœtales sont par ailleurs responsables de la descente des testicules en position scrotale *via* la synthèse et la sécrétion du facteur *Insulin-Like 3* (Insl3) (158).

D'un point de vue moléculaire, la différenciation testiculaire est initiée par l'expression du gène *Sex determination Region on Y chromosome* (Sry), localisé sur le chromosome Y (**Figure 11**). Elle enclenchera une cascade moléculaire conduisant à la différenciation des cellules de Sertoli qui constitue la première étape du programme de développement testiculaire (159)-(160)-(161). L'expression de Sry est transitoire et limitée aux précurseurs des cellules de Sertoli, dans lesquels elle débute à E10.5 et s'éteint après le stade embryonnaire E12.5 (162). Elle est responsable de l'induction de l'expression du facteur de transcription *Sry-related HMG box 9* (Sox9) (163), qui sera maintenue à un niveau élevé grâce au facteur de transcription Sf-1, et à une boucle de rétrocontrôle positive se mettant en place entre Sox9 et le facteur de croissance Fgf9. La signalisation associée au facteur de transcription Sox9 conduit à l'activation de gènes impliqués dans la différenciation des cellules de Sertoli. celles-ci synthétiseront alors des facteurs qui participeront à la différenciation des cellules de Leydig (*Desert Hedgehog* (Dhh), *Plateled-derived growth factor* (Pdgf)). Sox9 inhibe par ailleurs, *via* Fgf9, la voie de signalisation *Wingless-type MMTV integration site family, member 4* (Wnt4) / R-Spondin-1 (Rspo1) impliquée dans la différenciation ovarienne (164).

2. Le développement pubertaire

2.1. Les cellules de Leydig

Au cours de la première semaine suivant la naissance, les cellules de Leydig fœtales entament leur différenciation en cellules de Leydig adultes, dont le nombre va croître jusqu'à environ 1 mois (165)-(166). Contrairement aux cellules fœtales, les cellules de Leydig adultes nécessitent la présence des gonadotrophines pour leur différenciation, leur prolifération et pour le maintien de leur fonction (167). Elles assureront au début de la puberté, un pic hyper sécrétoire de testostérone en réponse à l'activation de la pulsatilité de l'hormone *Gonadotropin Releasing Hormone* (Gnrh) et à l'augmentation de la sécrétion des gonadotrophines Lh et Fsh par l'axe hypothalamo-hypophysaire. Les androgènes seront responsables de l'apparition des caractères sexuels secondaires, et participeront à l'initiation de la spermatogenèse.

2.2. Les cellules de Sertoli

Au cours de la puberté, les cellules de Sertoli, alors immatures, vont subir des changements morphologiques et fonctionnels majeurs afin d'acquérir leur maturité. A la naissance, les cellules de Sertoli reprennent une activité prolifératives intense durant les 3 premières semaines post-natales. L'arrêt de la prolifération des cellules de Sertoli est concomittant à un processus de différenciation dit terminal, caractérisé par des changements morphologiques, la production de protéines sécrétées nécessaires à la survie et à la différenciation des cellules germinales, et à l'établissement de la barrière hémato-testiculaire (BHT).

D'un point de vue morphologique, les cellules de Sertoli, de forme cuboïdale à la naissance, augmentent de taille et deviennent pyramidale. Leur noyau, à l'origine arrondi, migre vers leur pôle basal et s'allonge en prenant une forme triangulaire caractéristique des cellules de Sertoli adultes. Elles accumulent par ailleurs des organites intracellulaires tels que le réticulum endoplasmique et l'appareil de Golgi qui leur seront nécessaires pour leur activité sécrétrice (168).

En dehors de la période fœtale, les cellules de Sertoli connaissent un deuxième et dernier épisode prolifératif au cours de la période post-natale permettant d'établir leur nombre définitif. Celui-ci détermine le rendement de la spermatogenèse et la taille du testicule, car chaque cellule de Sertoli ne peut supporter qu'un nombre fixe de cellules germinales (169). La régulation du taux et de la durée de la prolifération des cellules de Sertoli est donc clée pour la fertilité. Certains acteurs impliqués dans cette régulation ont été identifiés.

La prolifération des cellules de Sertoli est en particulier favorisée par la Fsh. En effet, une élévation de la concentration de Fsh néo-natale par injections ou hémi-castration, entraîne une augmentation du nombre de cellules de Sertoli de 49% et 18% respectivement chez le rat (170)-(171). A l'inverse, les souris dont le gène codant la sous-unité β de la Fsh (FshβKO) ou son récepteur (FshrKO) a été invalidé sont fertiles, mais présentent une diminution du nombre de cellules germinales (172)-(173)-(174), associée à une diminution du nombre de cellules de Sertoli de près de 40% chez l'adulte (175). L'altération du nombre de cellules de Sertoli observée chez ces modèles animaux serait le résultat d'une perturbation du taux de prolifération par la Fsh, et non pas de la durée de cet épisode prolifératif, puisque les concentrations plasmatiques de Fsh, ainsi que l'expression de son récepteur dans les cellules de Sertoli, augmentent continuellement au cours du développement post-natal précoce (176). Ainsi, l'arrêt de la prolifération des cellules de Sertoli résulte-t-il en partie de l'action de l'hormone thyroïdienne triiodothyronine (T_3). En effet, une hypothyroïdie transitoire en période post-natale aboutit à une augmentation du taux et de la durée de la prolifération des cellules de Sertoli, conduisant à une élévation du nombre de ces cellules, de la production de spermatozoïdes et de la taille des testicules chez le rat (177)-(178) et la souris (179). L'hypothyroïdie juvénile est également associée à une macro-orchidie chez l'Homme (180). A l'inverse, une hyperthyroïdie causée par l'injection répétée de triiodothyronine est responsable d'un arrêt prématuré de la prolifération des cellules de Sertoli, et d'une canalisation précoce des tubes séminifères (181). D'un point de vue moléculaire, l'hormone T_3 agirait par l'intermédiaire de l'inhibiteur du cycle cellulaire $p27^{kip1}$. L'expression de cette *cyclin-dependent kinase inhibitor* (CDKI) dans les cellules de Sertoli est en effet inversement corrêlée à leur activité proliférative (182) ; de plus, son expression est diminuée chez des souris hypothyroïdiennes, et augmentée chez des souris hyperthyroïdiennes de 10 jours post-nataux par rapport aux animaux témoins. Enfin, la T_3, l'acide rétinoïque et la testostérone répriment

in vitro la prolifération de cellules de Sertoli de rat, et induisent l'expression de marqueurs de différenciation de ces cellules (GATA-1, transferrine) (183). L'action conjointe de ces trois acteurs seraient donc requise pour enclencher l'arrêt de la prolifération des cellules de sertoli et pour initier leur différenciation terminale, menant à l'acquisition de leur maturité fonctionnelle.

La différenciation terminale des cellules de Sertoli est caractérisée par la perte et / ou l'acquisition de l'expression de gènes spécifiques. On observe par exemple une diminution de l'expression du gène codant l'Amh, accompagnée d'une diminution de sa sécrétion dans le système sanguin. De même, l'expression de l'aromatase, enzyme catalysant la conversion de la testostérone en œstradiol, décline au cours de cette période. A l'inverse, les cellules de Sertoli acquièrent l'expression des gènes codant les protéines Gata-1, $p27^{kip1}$ et le récepteur nucléaire aux androgènes Ar (184). Enfin, l'expression de gènes codant des protéines de jonctions membranaires s'accentue et conduira à la formation de la barrière hémato-testiclaire. Il est intéressant de noter que l'invalidation spécifique dans les cellules de Sertoli des gènes codant certaines de ces protéines (connexine 43 et claudine 11) conduit à l'augmentation de la prolifération de ces cellules, suggérant que la formation de la BHT puisse être un facteur supplémentaire contrôlant l'activité proliférative des cellules de Sertoli (185)-(186).

Une fois arrivées à maturité, les cellules de Sertoli enclenchent la synthèse et la sécrétion de facteurs nécessaires à la survie et à la différenciation des cellules germinales (transferrine, *Androgen-Binding Protein* (Abp)...). La mise en route de cette activité sécrétrice conduira à la canalisation des cordons testiculaires qui seront dès lors appelés tubes séminifères. Elle serait par ailleurs dépendante de la Fsh, qui *via* l'activation du facteur de transcription *Krüppel-Like Factor 4* (Klf4), régulerait l'expression de gènes impliqués dans le transport vésiculaire et le processus d'exocytose nécessaire à la sécrétion apicale des cellules de Sertoli (187)-(188).

2.3. Les cellules germinales : la première vague de spermatogenèse

A 2-3 jours post-nataux, les gonocytes, qui étaient alors quiescents, reprennent leur activité mitotique et migrent vers la membrane basale des tubes séminifères où ils se différencieront en cellules germinales souches adultes (spermatogonies adultes A_{single}) (156). Celles-ci, grâce au soutien des cellules de Leydig et de Sertoli, initieront la première vague de spermatogenèse.

L'une des étapes critiques de la spermatogenèse est la méiose, dont l'initiation et la progression sont dépendantes de l'action des rétinoïdes. Ceux-ci sont maintenus présents à une faible concentration dans le testicule fœtal grâce à l'action catabolique de l'enzyme *Cytochrome P450, family 26, subfamily b, polypeptide 1* (Cyp26b1), empêchant ainsi l'entrée en méiose des cellules germinales (189)-(190). La puberté est caractérisée par la répression de l'expression de cette enzyme. On

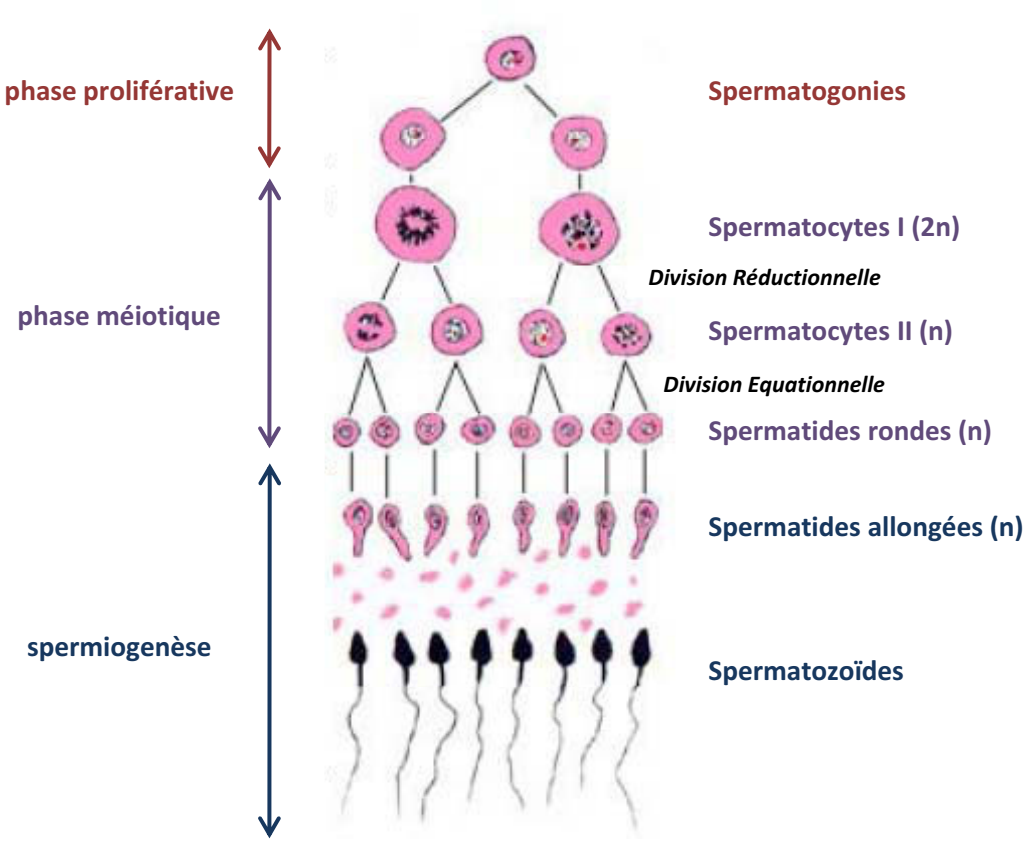


Figure 12 : Représentation schématique de la spermatogenèse. La spermatogenèse est divisée en trois phases, chacune caractérisée par un stade germinal particulier. Les spermatogonies s'amplifient par mitoses successives au cours de la phase prolifération, avant de se différencier en spermatocytes primaires pré-leptotènes. Ces derniers subiront les deux divisions méiotiques (réductionnelle puis équationnelle), aboutissant à la formation de spermatides haploïdes, qui donneront naissance aux spermatozoïdes au terme de la spermiogenèse.

observe alors une accumulation des rétinoïdes qui, *via* leur récepteur nucléaire *Retinoic Acid Receptor alpha* (Rarα, Nr1b1) induisent l'expression, par les cellules germinales, de *Stimulated by Retinoic Acid gene 8* (Stra8), acteur responsable de l'initiation de la méiose (191). D'autres gènes cibles germinaux de l'acide rétinoïque (*Disrupted Meiotic gene 1* (Dmc1), *Synaptonemal Complex Protein 3* (Sycp3)) sont quant-à eux impliqués dans la progression de la méiose (192)-(193)-(194). L'action des rétinoïdes est limitée par le récepteur nucléaire Shp par le biais de deux mécanismes indépendants. Il diminue d'une part la biodisponibilité de l'acide rétinoïque en stimulant l'expression de cyp26b1, et altère d'autre part l'activité transcriptionnelle du récepteur Rarα sur le promoteur de ses gènes cibles (33). Les souris $Shp^{-/-}$ présentent ainsi une entrée en méiose précoce des cellules germinales associée à des niveaux élevés des transcrits Stra8. L'action des rétinoïdes est également contrecarrée par le facteur de transcription spécifique aux gonades *Doublesex-Related and Mab-3 Transcription factor 1* (Dmrt1) qui contrôle la transition mitose-méiose (195).

La spermatogenèse est ainsi initiée dès l'âge de 8-10 jours chez la souris. Cette première vague est caractérisée par une apoptose massive des cellules germinales, indispensable pour le bon déroulement des futurs cycles de spermatogenèse (196). En effet, la prévention de cette vague apoptotique précoce conduit à des altérations majeures de la spermatogenèse et à la stérilité des animaux adultes (196). Ce processus physiologique permettrait d'adapter le nombre de cellules germinales au nombre de cellules de Sertoli, capables de n'assurer le soutien structural et nutritionnel que d'un nombre limité de cellules germinales.

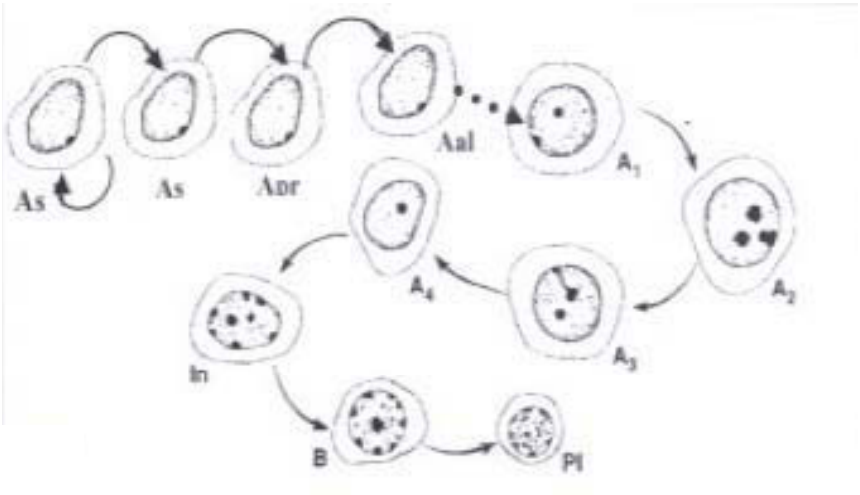
Les premiers spermatozoïdes sont détectés dans la lumière des tubes séminifères aux environ de 40-45 jours post-nataux. Le testicule est alors considéré comme fonctionnel et les cycles de spermatogenèse s'enchaînent.

B. Le testicule adulte

1. La lignée germinale : la spermatogenèse

La spermatogenèse est un processus complexe et hautement coordonné aboutissant à la production de gamètes mâles différenciés à n chromosomes à partir de cellules germinales souches indifférenciées à 2n chromosomes. Elle peut se diviser en trois phases, chacune caractérisée par un stade germinal particulier (**Figure 12**) : 1. la phase prolifératives, au cours de laquelle les spermatogonies s'amplifient à travers une série de divisions mitotiques, avant de se différencier en spermatocytes pré-méiotiques ; 2. la phase méiotique caractérisée par la réduction du matériel génétique aboutissant à la formation de spermatides haploïdes ; 3. la maturation des spermatides au

A



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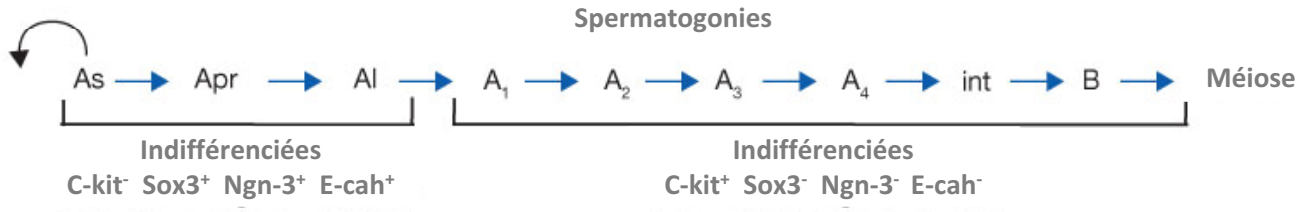

Figure 13 : La phase prolifération de la spermatogenèse. A. Représentation schématique de la phase prolifération de la spermatogenèse. La spermatogenèse repose sur une population de cellules germinales souches, les spermatogonies de type A_s. Elles ont la capacité de s'auto-renouveler, maintenant ainsi un pool de cellules A_s, ou de se différencier en progéniteurs qui s'engageront dans la voie de différenciation germinale. Ceux-ci s'amplifient par mitoses, donnant naissance à une chaîne de 2 (spermatogonies A_{pr}) puis 4, 8 et 16 (spermatogonies A_{al}) cellules filles. Les spermatogonies A_{al} se différencient alors en spermatogonies de type A_1 qui subiront 6 divisions mitotiques supplémentaires et se différencieront à leur tour en spermatogonies A_2, A_3, A_4, intermédiaire puis B pour former les spermatocytes pré-leptotènes qui subiront la méiose. **B.** Diagramme reflétant le profil d'expression de marqueurs de différenciation des spermatogonies. On distingue les spermatogonies indifférenciées (A_s, A_{pr}, A_{al}) des spermatogonies différenciées (A_1,A_2, A_3, A_4, intermédiaire et B) en fonction de l'expression des marqueurs de différenciation c-kit, Sox3, Ngn-3 et E-cadh (Barroca *et al.*, 2008).
A_s : A_{single} ; A_{pr} : A_{paired} ; A_{al} : $A_{aligned}$; Sox3 : Sry-related HMG box 3; Ngn-3 : Neurogenin-3 ; E-cadh : E-cadherin.

cours du processus de spermiogenèse aboutissant à la formation des spermatozoïdes testiculaires immatures.

La durée de la spermatogenèse est variable selon les espèces. Chez la souris et l'Homme elle est respectivement de 35 et 74 jours. C'est un processus cyclique, avec un nouveau cycle initié tous les 8,6 jours chez la souris (197). A chaque nouveau cycle de spermatogenèse, les cellules germinales néo-formées repoussent les générations précédentes vers la lumière des tubes, formant ainsi des associations cellulaires représentant les 12 stades de l'épithélium séminifère (I-XII) chez la souris. Les cycles sont initiés de façon asynchrone sur toute la longueur des tubes séminifères. Les différents stades de l'épithélium séminifère sont de ce fait présents à différentes positions du tube, permettant une production continue de spermatozoïdes.

1.1. La phase prolifération

1.1.a. Les spermatogonies indifférenciées

La spermatogenèse débute avec la différenciation des cellules germinales souches (ou *Spermatogonial Stem Cells*, SSCs), localisées au contact de la membrane basale des tubes séminifères, et qui assurent une spermatogenèse continue tout au long de la vie d'un individu. Initialement, les spermatogonies de type A_{single} ont été identifiées comme étant les SSCs chez les mammifères non primates (198)**-**(199) ; mais une étude plus récente a montré que cette population de cellules est hétérogène d'un point de vue fonctionnel, et que seul 12% d'entre elles (représentant environ 3000 SSCs soit 0,01% du nombre total de cellules dans un testicule adulte) sont de ''vraies'' SSCs capables de recoloniser un testicule receveur vidé de son contenu germinal et de régénérer de manière durable l'épithélium séminifère (200). Au cours de la phase proliférative (**Figure 13**), les SSCs se multiplient pour soit renouveler la population de SSCs donnant ainsi naissance à de nouvelles spermatogonies de type A_{single}, soit générer une population de progéniteurs qui s'engageront dans la voie de différenciation germinale ; elles entreprennent dans ce deuxième cas quatre divisions mitotiques successives donnant ainsi naissance à 2 spermatogonies de type A_{paired} puis à une chaîne de 4, 8 et 16 (et plus rarement 32) spermatogonies de type $A_{aligned}$ (201). L'ensemble de ces cellules filles issues d'une même SSC restent interconnectées tout au long de la phase proliférative par le biais de ponts intercellulaires qui permettent le transfert de substances (ARN messagers, protéines) dont l'action synchrone assure la coordination des cellules germinales tout au long de leur maturation (202). Ces trois types de spermatogonies (A_{single}, A_{paired} et $A_{aligned}$) constituent les spermatogonies indifférenciées sur la base de critères morphologiques, et se distinguent les unes des autres par leur arrangement topographique le long de la membrane basale, et par leur profil d'expression.

1.1.b. Les spermatogonies différenciées

Les spermatogonies indifférenciées prolifèrent durant une partie du cycle de l'épithélium séminifère (stades X-II) avant de devenir quiescentes (stades III-VII). Au stade suivant (VIII) les spermatogonies $A_{aligned}$ se différencient en spermatogonies de type A_1 qui subiront 6 divisions mitotiques supplémentaires et se différencieront à leur tour en spermatogonies de type A_2, A_3, A_4, intermédiaire puis B pour former les spermatocytes pré-leptotènes qui subiront la méiose (201). L'ensemble de ces spermatogonies forment les spermatogonies différenciées.

Les spermatogonies indifférenciées (A_{single}, A_{paired} et $A_{aligned}$) et différenciées (A_1, A_2, A_3, A_4, intermédiaire et B) sont discernables les unes des autres de part l'expression de certains gènes : les cellules indifférenciées n'expriment pas le récepteur tyrosine kinase *c-kit*, mais expriment les facteurs de transcription *Neurogenin-3* (Ngn-3) et *Sox3,* ainsi que la *E-cadherin* (E-cadh) contrairement aux spermatogonies différenciées (203)-(204)-(205)-(206).

1.1.c. Régulation de la différenciation des spermatogonies

Deux étapes de différenciation ont donc lieu au cours de cette phase proliférative : 1. la première correspond à la différenciation des spermatogonies de type A_{single} en A_{paired}, soit au passage des SSCs à des progéniteurs prédestinés à s'engager dans la différenciation germinale ; 2. la deuxième correspond à la différenciation des spermatogonies de type $A_{aligned}$ en A_1, soit au passage des spermatogonies indifférenciées aux spermatogonies différenciées.

i. Régulation de la transition A_{single} - A_{paired}

La balance entre auto-renouvellement et différenciation des SSCs est essentielle pour le maintien de vagues cycliques de spermatogenèse et de la fertilité. Dans l'épithélium séminifère adulte normal, la moitié des divisions des SSCs génèrent d'autres SSCs pour maintenir le nombre de ces cellules, tandis que l'autre moitié donne naissance à des spermatogonies de type A_{paired} qui deviendront des spermatozoïdes à l'issue de leur différenciation. Dans le cas où l'auto-renouvellement dominerait, l'épithélium séminifère se résumerait aux cellules souches et des tumeurs pourraient se former ; à l'inverse, si la différenciation était favorisée, il y aurait une déplétion des cellules germinales souches et une perte progressive de la lignée germinale. Il est intéressant de noter que le testicule est capable d'adapter ce ratio auto-renouvellement / différenciation en réponse à certaines situations pathologiques. Suite à l'exposition du testicule à des molécules reprotoxiques ou à une irradiation, on assiste à une perte totale de la lignée germinale ; l'auto-renouvellement des SSCs survivantes prend alors le pas sur la différenciation, permettant de régénérer l'épithélium séminifère (207)-(208).

Les SSCs évoluent dans un micro-environnement spécialisé appelé niche, créé en partie par les cellules somatiques adjacentes, en particulier par les cellules de Sertoli. Celles-ci assurent un support physique et la sécrétion de facteurs solubles nécessaires à la régulation de la balance auto-renouvellement / différenciation des SSCs. Parmi ces derniers, nous pouvons citer le facteur de croissance *Glial cell Derived Neurotrophic Factor* (Gdnf) et le facteur de transcription *Ets Related Molecule* (Erm) (209)-(210). En effet, bien que la première vague de spermatogenèse se déroule normalement, les testicules de souris $Gdnf^{+/-}$ ou $Erm^{-/-}$ montrent une déplétion progressive de la lignée germinale dès 6 semaines. Elle est le résultat d'une perte des spermatogonies, et aboutit à l'observation de tubes séminifères composés exclusivement de cellules de Sertoli et à la stérilité. A l'inverse, les souris sur-exprimant le facteur Gdnf montrent une accumulation de spermatogonies indifférenciées et la formation de tumeurs de cellules germinales. Gdnf agit sur les SSCs par l'intermédiaire d'un complexe de récepteurs c-Ret / *GDNF Family Receptor alpha 1* (Gfrα1) (211). L'invalidation de ces deux partenaires conduit à un phénotype similaire à celui observé chez les souris $Gdnf^{+/-}$, soulignant d'avantage l'importance de la signalisation Gdnf dans le contrôle de la balance auto-renouvellement / différenciation des SSCs (212).

Des facteurs intrinsèques aux spermatogonies sont également impliqués dans le maintien de la balance auto-renouvellement / différenciation des SSCs, parmi lesquels le répresseur transcriptionnel *promyelocytic Leukemia Zinc Finger protein* (Plzf) (213). La perte d'expression de Plzf se traduit en effet par une perte progressive des spermatogonies, conduisant à l'observation de tubes séminifères vidés de leur contenu germinal et à un phénotype d'infertilité (214)-(215). De plus, les SSCs issues de souris déficientes pour Plzf sont incapables de coloniser un testicule receveur après transplantation, démontrant que l'action de Plzf est intrinsèque aux SSCs. Par ailleurs, l'invalidation du gène codant le facteur *TATA box–binding protein (TBP)-associated factor 4b* (Taf4b) se traduit également par un épuisement de la lignée germinale, et les mâles deviennent stériles dès l'âge de trois mois (216). Ces facteurs favoriseraient donc l'auto-renouvellement des SSCs et / ou inhiberaient leur capacité de différenciation.

ii. Régulation de la transition $A_{aligned}$ - A_1

La différenciation des spermatogonies indifférenciées en spermatogonies différenciées met en jeu notamment l'acide rétinoïque. En effet, un déficit en vitamine A entraîne un blocage de la spermatogenèse spécifiquement au stade spermatogonies $A_{aligned}$ qui, alors incapables de se différencier, sont bloquées dans un état quiescent (217)-(218). L'action de l'acide rétinoïque pourrait être directe ou indirecte car ses récepteurs sont exprimés à la fois par les spermatogonies et par les cellules de Sertoli (219)-(220). Le récepteur tyrosine kinase c-kit et son ligand *Steel Factor* (Slf) ou

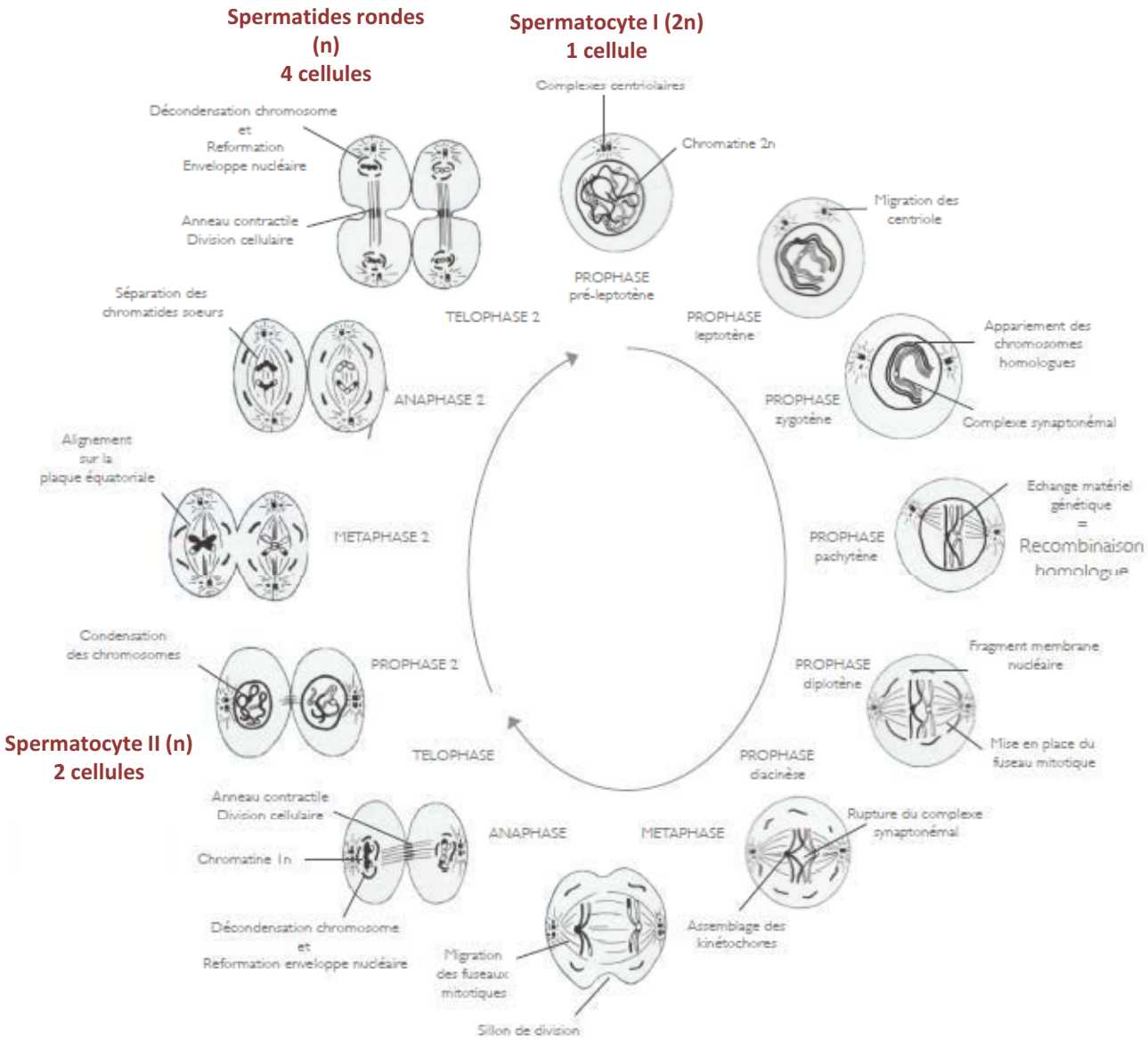

Figure 14 : Représentation schématique de la méiose. La méiose consiste en la formation de quatre cellules filles haploïdes à n chromosomes simples (spermatides) à partir d'une cellule mère diploïde à 2n chromosomes à deux chromatides (spermatocyte I). Elle est le résultat de deux divisions successives, chacune décomposée en cinq phases : prophase, métaphase, anaphase, télophase et cytodiérèse. La prophase de la première division est particulièrement longue, et est marquée par la recombinaison homologue qui permet le brassage de l'information génétique. La première division, dite réductionnelle, permet la formation des spermatocytes II à n chromosomes à deux chromatides. Chacune d'elle sera répartie dans les deux spermatides issues de la deuxième division, dite équationnelle.

Stem Cell Factor (Scf), ainsi que la protéine de liaison aux ARN *Deleted in azoospermia-like* (Dazl), sont également impliqués : les souris mutées pour Slf (souris *Sl17H/Sl17H*) ou déficientes pour Dazl sont en effet stériles du fait d'un défaut de différenciation des spermatogonies $A_{aligned}$ en spermatogonies A_1 (221)-(222). Enfin, une étude s'intéressant à l'expression des cyclines D dans le testicule murin en développement et le testicule adulte, a montré que la cycline D2 commence à s'exprimer au démarrage de la spermatogenèse lorsque certains gonocytes se différencient en spermatogonies de type A_1. Dans le testicule adulte, elle est exprimée uniquement autour du stade VIII de l'épithélium séminifère qui correspond au moment où les spermatogonies de type $A_{aligned}$ se différencient en spermatogonies A_1, suggérant un rôle de la cycline D2 dans ce processus de différenciation. Cette hypothèse est d'avantage soutenue par le fait que la cycline D2 n'est pas détectée dans les spermatogonies indifférenciées chez les souris déficientes en vitamine A, tandis que son expression est induite dans certaines de ces cellules lorsque la différenciation des spermatogonies $A_{aligned}$ en spermatogonies A_1 est permise par une supplémentation en acide rétinoïque (223).

1.2. La méiose

La méiose est une division cellulaire particulière impliquée dans la reproduction sexuée des organismes, aboutissant à la production des cellules sexuelles au cours de la gamétogenèse. Elle se déroule en plusieurs étapes formant un ensemble de deux divisions cellulaires, successives et inséparables, précédées d'une unique phase de réplication. Elle aboutit à la formation de quatre cellules filles haploïdes à n chromosomes simples à partir d'une cellule mère diploïde à 2n chromosomes homologues à deux chromatides (**Figure 14**).

Chacune des deux divisions méiotiques se compose de cinq phases : prophase, métaphase, anaphase, télophase et cytodiérèse. La prophase de première division est une étape particulièrement longue, couvrant à elle seule 90% de la durée totale de la méiose, et peut être subdivisée en cinq étapes correspondant à cinq états caractéristiques de la chromatine : leptotène, zygotène, pachytène, diplotène et diacinèse. Elle est par ailleurs une étape primordiale de la méiose car marquée par deux évènements majeurs. Ainsi, les spermatocytes leptotènes migrent-ils du compartiment basal vers le compartiment apical du tube séminifère, grâce à une restructuration transitoire de la barrière hémato-testiculaire, afin de préparer la méiose (224). C'est également au cours de la prophase I, au stade pachytène, qu'ont lieu les échanges de matériel génétique entre les chromatides des chromosomes homologues appariés participant ainsi au brassage de l'information génétique. La première division méiotique est dite réductionnelle car elle permet de passer d'une

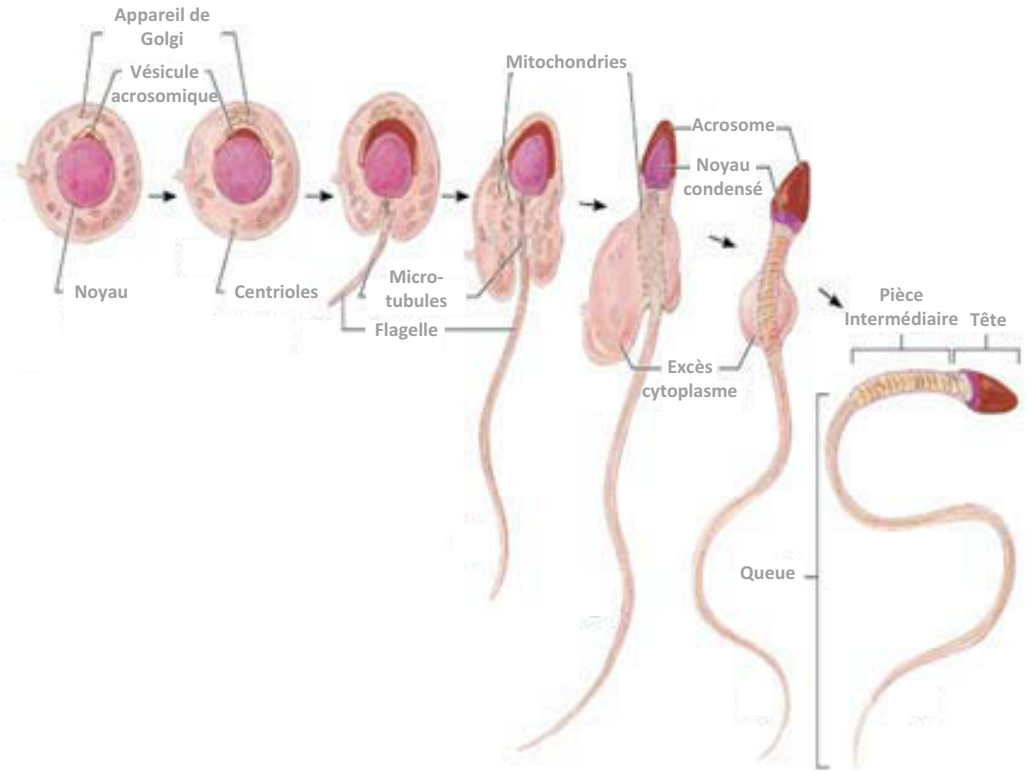

Figure 15 : La spermiogenèse. La spermiogenèse est le processus de différenciation terminale de la spermatogenèse aboutissant à la formation de spermatozoïdes allongés à partir de spermatides rondes. Elle est caractérisée par plusieurs changements biochimiques et morphologiques progressifs majeures de la cellule germinale. Les histones sont remplacées par des protéines de transition, puis par les protamines conduisant à la compaction de la chromatine et la condensation du noyau. L'acrosome se forme à partir de l'appareil de Golgi et recouvre progressivement le noyau spermatique. Le centriole distale s'allonge pour former l'axonème, qui est la structure axiale du flagelle et le véritable moteur du spermatozoïde. Les mitochondries se regroupent autour de la partie proximale de l'axonème, pendant que le cytoplasme migre du pôle céphalique vers le pôle caudal du futur spermatozoïde le long du flagelle en croissance. Il sera éliminé sous la forme de corps résiduels ensuite phagocytés par les cellules de Sertoli.

cellule mère (spermatocyte primaire) à 2n chromosomes à deux chromatides, à deux cellules filles (spermatocytes secondaires) à n chromosomes à deux chromatides.

La deuxième division de méiose se déroule immédiatement après la première : chaque chromosome étant resté dupliqué, il n'y a en effet pas de cycle de réplication de l'ADN supplémentaire. Elle consiste en une simple mitose classique, et est donc qualifiée d'équationnelle car elle permet de passer d'une cellule (spermatocyte secondaire) à n chromosomes à deux chromatides, à deux cellules (spermatides rondes) à n chromosomes à une chromatide.

1.3. La spermiogenèse

La spermiogenèse est le processus de différenciation terminale de la spermatogenèse au cours duquel les spermatides rondes haploïdes subissent plusieurs changements biochimiques et morphologiques, qui peuvent se dérouler de manière synchrone, conduisant à la formation des spermatozoïdes : compaction de la chromatine, développement de l'acrosome et du flagelle, élimination du corps résiduel (**Figure 15**).

Chez la souris, la spermiogenèse a été décomposée en 16 étapes développementales selon des critères morphologiques (197). On distingue ainsi les spermatides précoces avec un noyau arrondi (stades 1 à 8) transcriptionnellement actif, permettant la synthèse des transcrits codant des protéines nécessaires à la poursuite de la différenciation, telles que les protéines de transition nucléaire (Tnp, *Transition Nuclear Protein*) et les protamines (Prm) (225). Puis les spermatides s'allongent progressivement (stades 9 à 11), tandis que leur chromatine se compacte de plus en plus (stades 12 à 14). Les spermatides matures présentent un noyau extrêmement condensé le rendant transcriptionnellement inactif ; la majorité de leur cytoplasme a été éliminée, et l'acrosome et le flagelle se sont formés (stades 15 à 16).

1.3.a. La compaction du noyau

Au cours de la spermiogenèse, le noyau des spermatides va subir une réorganisation morphologique et biochimique conduisant à son allongement et à la compaction de la chromatine (226). Celle-ci est le résultat du remplacement de la majorité des histones par de petites protéines basiques dites de transition (Tnp1 et 2) qui seront remplacées à leur tour par des protéines nucléaires spécifiques au testicule, les protamines (Prm1 et 2) (225). Celles-ci sont particulièrement riches en résidus cystéine qui leur permettront d'établir des ponts disulfure afin de les maintenir étroitement associées les unes aux autres conduisant à un état de compaction maximale de la chromatine. Le génome devient ainsi moins perméable et donc moins vulnérable aux agressions physiques et chimiques générées en présence d'agents mutagènes, préservant de ce fait l'intégrité de l'information génétique tout au

long de la progression des spermatozoïdes dans les voies génitales mâles et femelles jusqu'à la fécondation (227). L'intégration des protamines, associée aux histones résiduelles (moins de 5%), participent par ailleurs à la constitution d'un code épigénétique à part entière essentiel à l'établissement du programme transcriptionnel du zygote lors des premières étapes de développement.

1.3.b. La formation de l'acrosome

L'acrosome est un organite localisé dans la tête du spermatozoïde, et dont la membrane enveloppe partiellement le noyau spermatique. Il se forme à partir de l'appareil de Golgi. Celui-ci produit des vésicules pro-acrosomiques et des granules qui au cours de la spermiogenèse vont fusionner pour former un unique acrosome accolé au noyau spermatique (228). Il est très riche en enzymes hydrolytiques (hyaluronidase, proacrosine...) qu'il libèrera au contact de la zone pellucide de l'ovocyte afin de la dégrader et d'ainsi permettre au spermatozoïde d'atteindre la membrane plasmique du gamète femelle. La réaction acrosomique permet par ailleurs de découvrir des récepteurs impliqués dans la liaison avec l'ovule et donc indispensables à la fécondation (229).

1.3.c. La formation du flagelle

Le flagelle est une structure essentielle assurant la mobilité du spermatozoïde en le propulsant grâce à un mouvement ondulatoire. Il se développe dès le début de la spermiogenèse à partir de deux centrioles, petites structures cylindriques perpendiculaires l'une à l'autre, et localisées initialement à proximité de l'appareil de Golgi. C'est à partir du centriole distal que se forme l'axonème, structure axiale du flagelle, composé de neuf doublets de microtubules disposés de manière circulaire autour d'une paire centrale de microtubules, et connectés entre eux par des liens de nexine. L'axonème est le véritable appareil moteur du spermatozoïde assurant sa mobilité tridimensionnelle. Il est entouré des structures périaxonémales qui modulent sa courbure (fibres denses externes et gaine fibreuse), et fournissent l'énergie pour les mouvements du flagelle (gaine de mitochondries).

1.3.d. Elimination du corps résiduel

Au cours de la spermiogenèse, la spermatide ronde va progressivement s'allonger et réduire son cytoplasme au minimum. Celui-ci va migrer du pôle céphalique vers le pôle caudal du futur spermatozoïde le long du flagelle en croissance, emportant avec lui les organites cytoplasmiques. Il finira par former une goutelette cytoplasmique qui va se détacher du flagelle pour former le corps résiduel qui sera éliminé par phagocytose par les cellules de Sertoli (230).

1.4. Spermiation

La spermatogenèse s'achève par le relargage des spermatozoïdes dans la lumière des tubes séminifères. Ce processus appelé spermiation est le résultat d'une restructuration des jonctions intercellulaires reliant les spermatozoïdes à leurs cellules de soutien, les cellules de Sertoli (231).

A la sortie du testicule, les spermatozoïdes ne sont pas matures : ils ne sont ni mobiles ni fécondants, propriétés qu'ils acquièreront au cours de leur transit épididymaire par le biais de modifications biochimiques importantes. Par ailleurs, leur chromatine continue à se condenser par la poursuite de la formation de ponts disulfures entre les protamines (232). Le génome du spermatozoïde étant donc à ce stade transcriptionnellement inactif, la maturation et la survie du gamète reposent exclusivement sur son interaction avec le milieu épididymaire, entièrement contrôlé par les cellules épithéliales de cet organe. Celles-ci sécrètent de nombreuses protéines spécifiques, dont la plupart sont des enzymes (phosphatases, protéases et inhibiteurs de protéases, glucosidases et glycosyltransférases), qui modifieront non seulement le milieu environnant des spermatozoïdes mais aussi leur revêtement membranaire. Ces modifications aboutissent notamment à l'apparition de récepteurs à la zone pellucide et à la membrane plasmique de l'ovocyte, nécessaires au processus de fécondation (233).

La maturation des spermatozoïdes s'achève au cours de leur ascension dans les voies génitales femelles par un processus appelé capacitation. Il est caractérisé par de nouvelles modifications membranaires qui mènent à l'hyperactivité des spermatozoïdes, et qui sont requises pour la future réaction acrosomique et la fécondation (234).

2. Les cellules de Sertoli

D'un point de vue morphologique, les cellules de Sertoli sont de forme pyramidale, et s'étendent de la lame basale jusqu'au compartiment apical des tubes séminifères. Leur très grande surface leur permet d'établir, *via* leur face latérale, un contact étroit avec les cellules de Sertoli adjacentes et avec un nombre important de cellules germinales : chez le rat, chaque cellule de Sertoli peut soutenir jusqu'à 50 cellules germinales à des stades de maturation différents (235). Les cellules de Sertoli accomplissent une série de fonctions essentielles au déroulement de la spermatogenèse : elles enserrent les cellules germinales, assurant un support structural et nutritif (grâce à leur activité sécrétice) pour leur différenciation, elles supportent une activité endocrine, elles créent une barrière perméable et immunologique (la BHT), elles participent au déplacement des cellules germinales le long de l'épithélium germinal et à la spermiation, et elles assurent l'élimination des corps résiduels issus de la spermiogenèse et des cellules germinales apoptotiques par phagocytose (236).

2.1. Fonctions

2.1.a. Support structural

Les cellules de Sertoli sont caractérisées par un cytosquelette très developpé. Composé principalement d'actine, de filaments intermédiaires et de microtubules, il est impliqué dans le maintien de l'intégrité de l'épithélium séminifère, il participe à l'établissement des jonctions membranaires inter-cellulaires, et il aide au mouvement des cellules germinales en différenciation et au relargage des spermatides matures dans la lumière des tubes séminifères au cours de la spermiation (236). Les cellules de Sertoli sont par ailleurs responsables, en coopération avec les cellules myoïdes péritubulaires, de la sécrétion et du dépôt d'éléments de la matrice extracellulaire (MEC), ciment de l'épithélium séminifère dans lequel évolue les cellules germinales (237).

2.1.b. Etablissement de la barrière hémato-testiculaire

L'une des fonctions majeures des cellules de Sertoli est la mise en place de la barrière hémato-testiculaire, observée au moment de la puberté sous l'action des androgènes, des rétinoïdes et des hormones thyroïdiennes (238)-(239)-(240). La BHT est composée d'un ensemble de jonctions spécialisées s'établissant entre les cellules de Sertoli adjacentes à proximité de la membrane basale des tubes séminifères : jonctions serrées (occludines, claudines, *Zonula Occludens*), jonctions adhérentes (cadhérines, caténines, nectines, laminines, intégrines), jonctions communicantes (connexines) (236). La BHT divise les tubes séminifères en un compartiment basal et un compartiment apical (adluminal). L'auto-renouvellement et la différenciation des spermatogonies, ainsi que la progression du cycle cellulaire jusqu'au stade spermatocyte pré-leptotène ont lieu dans le compartiment basal, tandis que les deux divisions méiotiques, la spermiogenèse et la spermiation se déroulent dans le compartiment apical (241).

La BHT assure plusieurs fonctions fondamentales au sein de l'épithélium séminifère (242). Les tubes séminifères n'étant pas traversés par les vaisseaux sanguins ou lymphatiques localisés dans le tissu interstitiel, les cellules de Sertoli régulent et restreignent le passage de substances nutritionnelles (sucres, acides aminés...), de molécules vitales (hormones, électrolytes...) et de composés toxiques (substances médicamenteuses, produits chimiques...) dans le compartiment apical des tubes séminifères. La BHT délimite par ailleurs un environnement de privilège immunitaire : elle isole en effet les nombreux antigènes présents spécifiquement à la surface des cellules germinales post-méiotiques de la circulation systémique, prévenant ainsi l'apparition des anticorps correspondants et de maladies auto-immunes à l'origine d'une stérilité (243). La BHT sépare enfin les cellules de Sertoli en deux pôles, basal et apical, respectivement au contact de la lame basale et de la lumière des tubes séminifère, leur conférant ainsi une polarité structurale et fonctionnelle. L'ensemble de ces fonctions permet à la BHT de créer un microenvironnement spécialisé nécessaire au développement post-

méiotique des cellules germinales. L'importance de l'intégrité de la BHT pour le soutien de la spermatogenèse est indéniable au vu notamment de modèles animaux adultes exposés par injections intra-péritonéales ou intra-testiculaires à diverses substances (cadmium, glycérol...) conduisant à une rupture de la BHT, accompagnée d'un blocage de la spermatogenèse (236).

Bien que la BHT soit l'une des barrière hémato-tissulaire la plus imperméable chez les mammifères, c'est une structure dynamique qui s'ouvre de façon régulière afin de faciliter le déplacement des cellules germinales au cours de la spermatogenèse. Les spermatocytes pré-leptotènes doivent en effet traverser la BHT à la fin du stade VIII-début du stade IX de l'épithélium séminifère afin de poursuivre leur différenciation dans le compartiment apical spécialisé (244). Ce processus implique que les jonctions intercellulaires Sertoli-Sertoli et Sertoli-germinales soient dissociées puis ré-assemblées périodiquement. De plus, il est particulièrement sélectif puisqu'aucun autre stade de différenciation germinal n'est autorisé à être transloqué d'un compartiment tubulaire à l'autre.

2.1.c. Fonction sécrétrice

Les cellules de Sertoli sont des cellules sécrétrices. Environ 15% des protéines totales synthétisées par les cellules de Sertoli sont destinées à être sécrétées, et peuvent être classées en plusieurs catégories : des composants de la matrice extracellulaire, des protéases et inhibiteurs de protéases, des substrats énergétiques, des facteurs de croissance, des facteurs paracrines et des hormones (236). La sécrétion des cellules de Sertoli est polarisée : une partie des sécrétions est déversée dans le compartiment basal des tubes séminifères, en direction de la lame basale et des spermatogonies ; une autre partie est dirigée vers le compartiment apical pour soutenir le développement méiotique et post-méiotique des cellules germinales, et pour alimenter le fluide séminifère qui permet la progression des spermatozoïdes dans la lumière des tubes (245).

i. Composants de la MEC, protéases et inhibiteurs de protéases

En lien avec leur rôle structural, les cellules de Sertoli participent à la sécrétion des composants de la matrice extracellulaire, indispensable au maintien de l'intégrité de l'épithélium séminifère et des jonctions membranaires inter-cellulaires (237). De plus, elles synthétisent et sécrètent des protéases et des inhibiteurs de protéases intervenant dans la dynamique des complexes de jonctions. Ces molécules facilitent ainsi la progression des cellules germinales au sein de l'épithélium et le relargage des spermatozoïdes au moment de la spermiation (236).

ii. Substrats énergétiques

Les cellules de Sertoli fournissent aux cellules germinales un ensemble d'éléments nutritifs essentiels à leur survie et à leur différenciation. Ces produits de synthèse comprennent notamment des acides aminés, des carbohydrates, des lipides et des vitamines (236). Les cellules de Sertoli sont par ailleurs capables de métaboliser le glucose en pyruvate, puis en lactate (246), ce dernier étant le substrat énergétique préférentiel des cellules germinales post-méiotiques pour soutenir leur activité métabolique (247)-(248). Le glucose circulant est capté par les cellules de Sertoli par l'intermédiaire de transporteurs spécifiques de type *Glucose Transporter* (Glut) avant d'être converti en pyruvate à l'issue de la glycolyse. Celui-ci est à son tour converti, sous l'action de la lactate déshydrogénase, en lactate, qui sera exporté des cellules de Sertoli par les transporteurs monocarboxylates avant d'être redistribué aux cellules germinales (249).

iii. Facteurs paracrines

Les cellules de Sertoli sécrètent de nombreux facteurs de croissance modulant la prolifération et / ou la différenciation des cellules germinales. Les facteurs Gdnf et Scf favorisent respectivement la prolifération des cellules germinales souches et la différenciation des spermatogonies de type $A_{aligned}$ en spermatogonies A_1 (209)-(221). Les facteurs *Insulin-like Growth Factor I* et *II* (Igf-I et Igf-II) et *Transforming Growth Factor α* (Tgf-α) favorisent quant-à eux *in vitro* la différenciation des spermatogonies en spermatocytes (250)-(251). Les cellules de Sertoli sécrètent par ailleurs de multiples protéines de transport qui assureront le passage des ions métalliques (Transferrine) (252), ou d'hormones (*Androgen Binding Protein*, protéine de liaison aux rétinoïdes) (253) à travers la BHT pour les besoins métaboliques des cellules germinales post-méiotiques.

iv. Activité endocrine

Les cellules de Sertoli sont des cellules endocrines à part entière, synthétisant et libérant dans la circulation systémique quelques hormones, appartenant pour la plupart à la famille du Tgfβ (Amh, inhibine, activine). Les cellules de Sertoli fœtales sécrètent l'Amh responsable de la régression des canaux de Müller. La sécrétion de l'Amh perdure jusqu'à la puberté, époque à laquelle elle disparaît sous le contrôle de la testostérone. Chez l'adulte, les cellules de Sertoli sécrètent deux glycoprotéines appartenant à cette même famille du Tgfβ : l'inhibine et l'activine (254)-(255). Ces deux hormones possèdent une activité biologique diamètralement opposée, l'inhibine réprimant la synthèse hypohysaire de la Fsh, tandis que l'activine la stimule (256)-(257). Un autre exemple de cette dualité d'action concerne leur effet local sur la stéroïdogenèse : en effet, l'inhibine potentialise l'effet de la Lh sur la production d'androgènes, alors qu'à l'inverse l'activine s'oppose à la stimulation de la biosynthèse des stéroïdes par la Lh *in vitro* (258)-(259).

2.1.d. Phagocytose

L'apoptose germinale est un processus physiologique indispensable au bon déroulement de la spermatogenèse. Elle permet en effet de contrôler la densité des cellules germinales, adaptant ainsi le nombre de ces cellules à celui des cellules de Sertoli (260)-(261). Elle participe également à l'élimination de toute cellule germinale présentant des anomalies de spermatogenèse (196)-(262). Les cellules de Sertoli assurent l'élimination de ces cellules germinales apoptotiques et des corps résiduels cytoplasmiques rejetés par les spermatides au cours de la spermiogenèse (230), par un processus de phagocytose. Cette fonction est nécessaire pour empêcher la libération de contenus toxiques, et constitue une source d'énergie importante pour les cellules de Sertoli.

Le processus de phagocytose débute par la reconnaissance de ''signaux apoptotiques'', que sont des résidus lipidiques Phosphatidylsérines présents à la surface des cellules en apoptose, par des récepteurs spécifiques affichés à la membrane des cellules de Sertoli. Deux récepteurs ont été identifiés : le récepteur Sr-b1 et le récepteur BAI1, points de départ de cascades de signalisation impliquant respectivement la phosphorylation et l'activation de la voie des Mapk, et l'activation du facteur *Engulfment and cell motility 1* (Elmo1), aboutissant à un réarrangement du cytosquelette d'actine nécessaires à l'internalisation des corps phagocytés (263)-(264).

2.2. Contrôle des fonctions Sertoliennes

2.2.a. Contrôle endocrine

i. La Fsh

La Fsh est le régulateur endocrinien majeur des fonctions Sertoliennes. Il s'agit d'une glycoprotéine appartenant à la famille des gonadotrophines. Sa synthèse et sa sécrétion par les cellules neuro-endocrines de l'hypophyse antérieure sont notamment sous la dépendance de l'hormone hypothalamique Gnrh (265). La Fsh exerce son action par l'intermédiaire d'un récepteur à 7 domaines trans-membranaires couplé aux protéines G, exprimé spécifiquement par les cellules de Sertoli dans le testicule (265). La fixation de la Fsh sur son récepteur est à l'origine de plusieurs voies de signalisation dans les cellules de Sertoli (266). La première à avoir été identifiée implique l'adénylate cyclase, responsable d'une augmentation de la production d'AMPc. Ce message intra-cellulaire conduit à l'activation successive de la *Protein Kinase A* (PkA) et du facteur de transcription *cAMP Response Element Binding Protein* (Creb) qui modulera l'expression d'un grand nombre de gènes. L'importance de cette voie de signalisation pour la survie germinale a été mise évidence en 2001 par l'étude de rats surexprimant spécifiquement dans les cellules de Sertoli une forme mutée non activable de Creb, et présentant une apoptose massive des spermatocytes conduisant à une

perte de plus de 75% des spermatides (267). Sous l'action de la Fsh, l'AMPc conduit en parallèle à un influx rapide de calcium permettant l'activation de *Calmodulin Kinases* (CamK) (268)-(269). Cette signalisation calcique joue un rôle dans l'intégrité structurale des cellules de Sertoli en intervenant dans le réarrangement de composants de leur cytosquelette (270), et en participant à la dynamique de leurs jonctions membranaires (271)-(236). De plus, l'augmentation des concentrations intracellulaires d'AMPc est à l'origine, *in vitro*, de l'activation de la voie *Phosphatidylinositol 3-Kinase / Protein Kinase B* (Pi3-k / PkB) impliquée dans la sécrétion de transferrine et la production de lactate (272). Enfin, la Fsh met en jeu la voie de signalisation des Mapk dans les cellules de Sertoli encore immatures.

La Fsh joue un rôle central dans le contrôle de la physiologie des cellules de Sertoli. Elle est un facteur prépondérant favorisant la prolifération de ces cellules au cours de la phase pré-pubère notamment par l'intermédiaire de la voie des Mapk (273). Elle participe ainsi à l'établissement du nombre de cellules de Sertoli adultes, et donc indirectement au rendement de la spermatogenèse. Les souris dont le gène codant la sous-unité β de la Fsh ou son récepteur, bien que fertiles, présentent en effet une baisse de la production germinale dûe à une diminution du nombre de cellules de Sertoli (172)-(173)-(174)-(175). Des anomalies qualitatives de la spermatogenèse ont par ailleurs été relevées chez ces animaux, démontrant un rôle clé de la Fsh dans la régulation des fonctions sertoliennes attitrées au soutien de la différenciation des cellules germinales (173). De façon cohérente avec le phénotype observé chez ces modèles animaux, cinq hommes chez lesquels une mutation inactivatrice du gène codant le récepteur à la Fsh a été identifiée sont fertiles, mais avec une diminution de la taille de leur testicule et une altération de la spermatogenèse (274). Ces données démontrent que la Fsh n'est pas indispensable pour la fertilité chez le mâle, mais est en revanche nécessaire pour supporter l'aspect quantitatif et qualitatif de la spermatogenèse.

Cette fonction est attribuée au soutien exercé par la Fsh sur l'activité globale des cellules de Sertoli. En effet, l'expression de près de 300 gènes sertoliens a été démontrée comme étant sous la dépendance de la Fsh (275). Parmi ceux-ci, de nombreux gènes ayant une implication directe dans le soutien de la spermatogenèse ont été identifiés : l'Abp (276), la lactate déshydrogénase (277), la transferrine (278), et les facteurs de croissance Igf-1 (279), Scf (280), Vegf (275), Gdnf (281) nécessaires au maintien du métabolisme des cellules germinales, ainsi qu'à leur prolifération et différenciation. La Fsh pourrait par ailleurs contrôler la survie des cellules germinales en stimulant la transcription de facteurs anti-apoptotiques (282)-(283). L'activité endocrine des cellules de Sertoli est également sous le contrôle de la Fsh au travers de la régulation de l'expression des gènes codant l'inhibine (284), l'activine (285) et l'aromatase (286). Citons également le facteur de transcription Dmrt1 impliqué dans la différenciation post-natale du testicule (287), et le récepteur de la Fsh (288).

Enfin, la Fsh augmente la transcription du gène codant le récepteur aux androgènes, et permet de cette façon de sensibiliser les cellules de Sertoli à la testostérone (289)-(290).

ii. Autres hormones

D'autres acteurs contribuent au contrôle hormonal du métabolisme des cellules de Sertoli. Comme cité précédemment dans ce manuscrit, l'hormone thyroïdienne est un acteur essentiel impliqué dans le développement post-natal des cellules de Sertoli, inhibant leur activité proliféatrice et favorisant leur différenciation terminale. D'un point de vue métabolique, la T_3 favorise la production de lactate par des cellules primaires de Sertoli isolées à partir de rats pré-pubères, mais n'a aucun effet sur des cellules issues d'animaux pubères (291). Ces résultats suggèrent que d'une façon générale, l'action de la T_3 sur les cellules de Sertoli est restreinte à la fenêtre de développement pubertaire.

L'activité métabolique Sertolienne est également sous la dépendance de l'insuline et du facteur de croissance Igf-1, dont les récepteurs sont exprimés par les cellules de Sertoli (292). La concentration élevée d'Igf-1 détectée dans le fluide interstitiel est en grande majorité le résultat de sa synthèse et de sa sécrétion hépatique (293). Cependant le facteur Igf-1 est également synthétisé localement par les principaux types cellulaires somatiques du testicule (Sertoli, péritubulaires et Leydig) (294)-(295)-(296)-(297). Igf-1 agit donc à la fois d'une manière endocrine, paracrine et autocrine sur les cellules de Sertoli. Des études *in vitro* ont démontré un effet stimulateur de l'insuline et de l'Igf-1 sur la synthèse d'ADN et la production de lactate par les cellules de Sertoli immatures (298)-(299), tandis qu'à l'inverse une privation en insuline se traduit par une altération de l'accumulation des transcrits de gènes impliqués dans la production (*lactate déhydrogenase*) et l'export (*monocarboxylate transporter* (Mct)) du lactate, dont la sécérétion se trouve diminuée (300). L'implication de l'insuline et d'Igf-1 dans la régulation de la physiologie Sertolienne a été récemment confirmée *in vivo* par l'étude de souris invalidées pour leur récepteur respectif spécifiquement dans les cellules de Sertoli (301). Ces animaux ''doubles mutants'' présentent une réduction de 75% de la taille du testicule et de la production de spermatozoïdes à l'âge adulte. Ce phénotype a été associé à une diminution du nombre de cellules de Sertoli dûe à une altération du taux de prolifération de ces cellules au cours de la période fœtale et néo-natale. Les auteurs ont en effet montré que la voie de signalisation insuline / Igf-1 était requise pour l'action pro-proliférative de la Fsh sur les cellules de Sertoli immatures. Ces données impliquant l'insuline pourraient expliquer en partie le lien observé entre les troubles de la fertilité masculine et le diabète.

2.2.b.Contrôle paracrine

En dehors de l'aspect endocrinien, l'activité des cellules de Sertoli est également sous la dépendance de tout un réseau de facteurs paracrines et autocrines, qui comptent parmi leur rang de nombreux facteurs de croissance, des cytokines ainsi que les androgènes.

i. Les facteurs de croissance

De nombreux facteurs de croissance sont synthétisés par les différents types cellulaires du testicule, parmi lesquels l'*epidermal growth factor* (Egf), le *fibroblast growth factor* (Fgf-I et Fgf-II), le *transforming growth factor* (Tgf-α et -β) et l'*insulin-like growth factor* (Igf-I et Igf-II), dont les récepteurs sont exprimés par les cellules de Sertoli (302). Des études *in vitro* sur des cellules de Sertoli isolées d'animaux pré-pubères ont démontré l'implication potentielle de certains de ces facteurs de croissance dans la régulation de la physiologie de ce type cellulaire. Le Fgf, l'Egf et l'Igf-1 ont ainsi une action mitogénique sur les cellules de Sertoli immatures (303)-(299), l'Igf-1 stimule la synthèse de lactate par les cellules de Sertoli (299), de même que l'Egf qui inhibe par ailleurs l'aromatisation des androgènes induite par la Fsh dans des cellules de Sertoli immatures (304).

ii. Les cytokines

Certaines molécules appartenant à la famille des cytokines, présentes dans le micro-environnement épithélial, sont également impliquées dans le contrôle du métabolisme des cellules de Sertoli. Parmi celles-ci, l'*Interleukin-1α* (Il-1α) est produite par différents types cellulaires testiculaires incluant les cellules de Sertoli adultes (305), les cellules germinales (306), ou encore les cellules de Leydig (307). l'Il-1α peut ainsi agir de manière autocrine et paracrine sur les cellules de Sertoli qui expriment son récepteur (308), altérant la production de transferrine et de lactate (309)-(310). De plus, la synthèse de ces protéines est stimulée par le *Tumor Necrosis Factor α* (Tnfα) libéré par les cellules germinales (311)-(312). Ces cytokines sont donc un exemple de la coopération métabolique s'établissant entre les cellules germinales et les cellules de Sertoli afin d'adapter l'activité de ces dernières aux besoins spécifiques de chacun des stades de différenciation germinale.

Ces deux cytokines montrent un profil d'expression dépendant des stades de l'épithélium séminifère à mettre en relation avec leur rôle dans la dynamique des jonctions inter-cellulaires (313)-(314). Des restructurations importantes sont réalisées au stade VIII de l'épithélium séminifère chez la souris, concernant d'une part les jonctions basales entre les cellules de Sertoli (BHT) pour permettre le transit des spermatocytes preleptotènes du compartiment basal vers le compartiment apical des tubes séminifères, et d'autre part les jonctions apicales entre les cellules de Sertoli et les spermatozoïdes nécessaires au processus de spermiation. Des études *in vivo* ont montré que l'injection intra-testiculaire de Tnfα et d'Il-1α chez le rat conduit à une rupture réversible de la BHT,

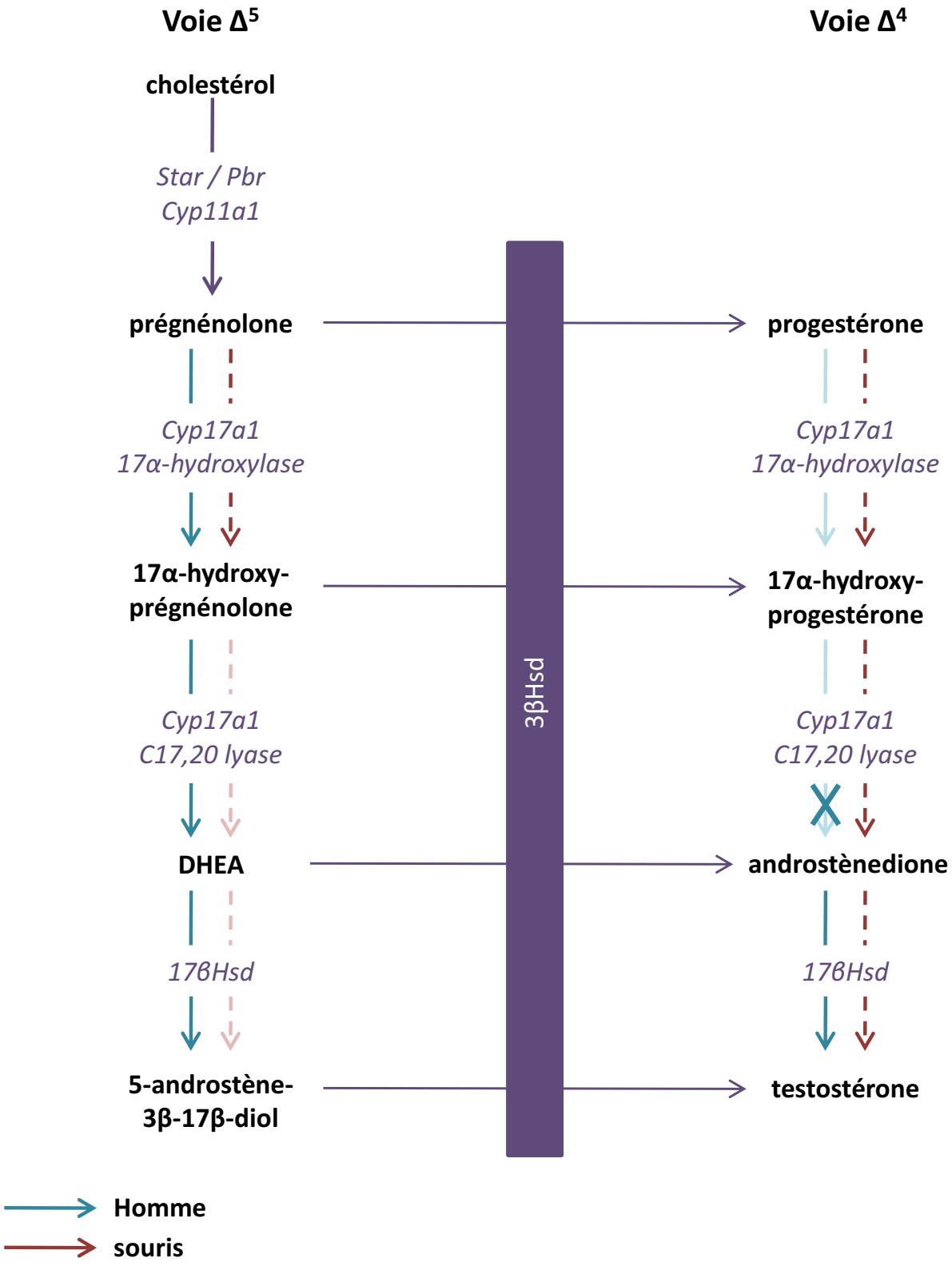

Figure 16 : Voies de biosynthèse des stéroïdes testiculaires. Dans les cellules de Leydig, la synthèse de testostérone à partir du cholestérol est assurée par deux voies possibles : la voie Δ^5 et la voie Δ^4. La voie Δ^5 est majoritaire chez l'Homme car l'enzyme CYP17A1 a une faible activité 17,20 lyase pour la conversion de la 17α-hydroxyprogestérone en androstènedione. A l'inverse, la voie Δ^4 est majoritaire chez la souris.
Star : Steroidogenic Acute Regulatory protein ; Pbr : Peripheral-type Benzodiazepine Receptor ; Cyp11a1 (P450scc) : cytochrome P450 side-chain cleavage ; 3βHsd : 3β-Hydroxysteroid Dehydrogenase ; Cyp17a1 : cytochrome P450 17α-hydroxylase / 17,20 lyase ; DHEA : déhydroépiandrostérone. Adapté de Scott et al. 2009.

associée respectivement à une diminution de l'immuno-détection des protéines de jonctions occludine, Zo-1 et N-cadhérine, et à une relocalisation des protéines occludine, Jam-1 et Zo-1 (315)-(316). Ces cytokines seraient donc un signal libéré par les cellules germinales, qui en coopération avec les androgènes, permettraient le remodelage transitoire des complexes de jonctions membranaires.

iii. Les androgènes

Les cellules de Sertoli commencent à exprimer le récepteur aux androgènes au cours du développement pré-pubère, puis son expression augmente avec l'âge (317). L'étude de modèles murins invalidés pour AR spécifiquement dans les cellules de Sertoli a révélé que la signalisation androgénique sertolienne est essentielle pour le développement méiotique et post-méiotique des cellules germinales, et pour la mise en place et le maintien de l'intégrité de la BHT (318)-(319)-(320)-(238)-(321).

3. Les cellules de Leydig

3.1. La biosynthèse des stéroïdes sexuels testiculaires

Chez les mammifères, la testostérone constitue la principale source d'androgènes circulants. Ceux-ci sont synthétisés en majeure partie (95%) par le testicule, cependant d'autres organes tels que les glandes surrénales sont capables d'en produire en petite quantité. Outre les androgènes, le testicule produit également de petites quantités d'œstrogènes et de progestérone. Ces différentes hormones stéroïdes partagent une voie de biosynthèse commune, avec pour unique précurseur le cholestérol. Celui-ci peut être synthétisé *de novo* par les cellules de Leydig, ou capté à partir des lipoprotéines de haute densité (HDL) circulantes (322).

La première étape enzymatique de la stéroïdogenèse consiste en la conversion du cholestérol en prégnénolone, catalysée au sein des mitochondries. Elle nécessite le transport du cholestérol de la membrane externe vers la membrane interne des mitochondries, étape limitante de la stéroïdogenèse, assuré par les protéines *Steroidogenic Acute Regulatory protein* (Star) (323) et *Peripheral-type Benzodiazepine Receptor* (Pbr) (324) (**Figure 16**). Le cholestérol est alors pris en charge par l'enzyme *cholesterol Side-Chain Cleavage* (cytochrome P450scc), codée par le gène Cyp11a1, qui catalyse le clivage de sa chaîne latérale conduisant à la formation de la prégnénolone (325). Celle-ci peut être convertie en testostérone par le biais de deux voies possibles (326). La première, nommée Delta 4 (Δ^4) implique la conversion de la prégnénolone en progestérone sous l'action de l'enzyme *3β-Hydroxysteroid Dehydrogenase* (3βHsd). La progestérone est alors métabolisée successivement par le *cytochrome P450 17α-hydroxylase / 17,20 lyase* (Cyp17a1) en

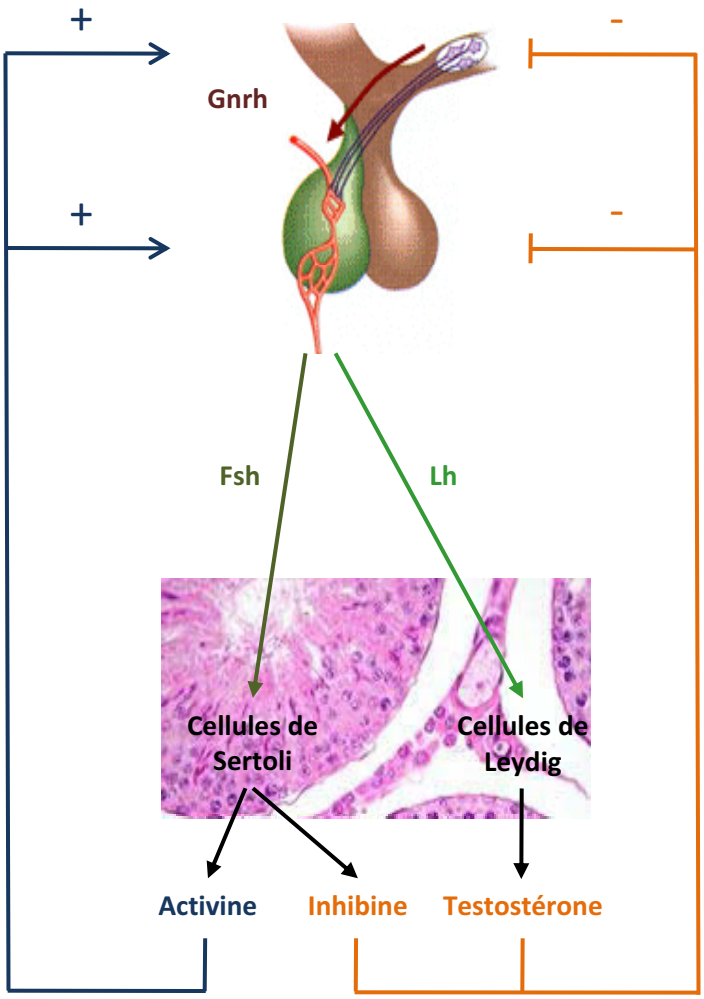

Figure 17 : Différents niveaux de contrôle de l'axe Hypothalamo-Hypophyso-Gonadique. La sécrétion des gonadotropines hypophysaires Lh et Fsh est sous la dépendance de la Gnrh synthétisée et sécrétée par l'Hypothalamus. La Lh agit directement sur les cellules de Leydig, permettant la production des stéroïdes sexuels qui exercent en retour un rétrocontrôle négatif sur leur propre synthèse en inhibant la synthèse hypothalamique de Gnrh et hypophysaire de la Lh et la Fsh. La Fsh gouverne les fonctions des cellules de Sertoli, en particulier la libération d'activine et d'inhibine, exerçant respectivement un contrôle positif et négatif sur l'axe Hypothalamo-Hypophysaire.
Gnrh : Gonadotropin Releasing Hormone ; Fsh : Follicle Stimulating Hormone ; Lh : Luteinizing Hormone.

17α-hydroxyprogestérone, puis en androstenedione, substrat de l'enzyme *17β-hydroxysteroid dehydrogenase* (17βHsd) pour la synthèse de testostérone. La seconde voie, dite Δ^5, est initiée par l'enzyme Cyp17a1 qui permet la conversion de la prégnénolone en 17α-hydroxyprégnénolone, elle-même convertie en déhydroépiandrostérone (DHEA). Les enzymes 17βHsd et 3βHsd assurent alors sa conversion en androstènediol ou androstènedione, puis en testostérone. L'enzyme 3βHsd permet de passer de la voie Δ^5 à la voie Δ^4. La contribution relative de ces voies Δ^4 et Δ^5 pour la stéroïdogenèse varie en fonction des espèces, et repose notamment sur le niveau d'expression des enzymes 3βHsd et Cyp17a1, et leur affinité relative pour la prégnénolone. Chez les primates dont l'Homme, et le bovin, la voie Δ^5 prédomine car l'enzyme Cyp17a1 a une faible activité 17,20 lyase pour la conversion de la 17α-hydroxyprogestérone en androstenedione (327)-(129)-(329).

3.2. Régulation de la synthèse de testostérone

Le maintien d'une concentration adéquate de testostérone intra-testiculaire est primordiale pour le bon déroulement des fonctions testiculaires, en particulier pour la spermatogenèse (330). La régulation de la stéroïdogenèse est donc essentielle. Elle fait intervenir un réseau complexe de signaux d'origines multiples, le principal étant la Lh. Par ailleurs, la testostérone régule sa propre synthèse d'une part en exerçant un rétrocontrôle négatif sur la synthèse et la sécrétion de la LH, et d'autre part en agissant de manière autocrine sur les cellules de Leydig. Enfin, la régulation de l'expression des gènes codant les enzymes de la stéroïdogenèse est également sous le contrôle de facteurs paracrines et de récepteurs nucléaires.

3.2.a. Régulation par l'axe hypothalamo-hypophysaire

L'axe hypothalamo-hypophysaire exerce un contrôle prépondérant sur la stéroïdogenèse testiculaire (**Figure 17**). La Gnrh est sécrétée par les neurones du noyau arqué de l'hypothalamus dans le sang porte hypophysaire, et stimule la synthèse et la libération des hormones Lh et Fsh par les cellules gonadotropes de l'hypophyse antérieure *via* son récepteur *GnRH Receptor* (Gnrh-R) (331). La Gnrh est libérée par l'hypothalamus sur un mode pulsatile, ce qui confère un caractère pulsatile et rythmique à la sécrétion des gonadotrophines hypophysaires. La Lh agit directement sur les cellules de Leydig afin de contrôler leur action stéroïdogène, tandis que la Fsh intervient indirectement dans cette régulation par l'intermédiaire de facteurs sécrétés par les cellules de Sertoli. Les stéroïdes sexuels exercent en retour un rétrocontrôle négatif sur leur propre synthèse en inhibant la sécrétion hypothalamique de Gnrh, et en réprimant la synthèse des hormones gonadotropes hypophysaires.

La Lh, en se fixant à son récepteur membranaire couplé aux protéines G présent à la membrane des cellules de Leydig, conduit à l'activation de l'adénylate cyclase et à la production du second messager

AMPc (332). Cela conduit notamment à l'activation, par phosphorylation par la PkA, du facteur de transcription Creb qui pourra ainsi activer la transcription de gènes cibles tels que Star (333), Cyp11a1 (334) et 3βHsd (335). La Lh intervient donc dans la régulation de la stéroïdogenèse à deux niveaux : en favorisant le transfert du cholestérol de la membrane externe vers la membrane interne de la mitochondrie, et en activant les systèmes enzymatiques responsables de la conversion du cholestérol en testostérone.

3.2.b. Régulation par les récepteurs nucléaires

L'expression et l'activité des enzymes impliquées dans la stéroïdogenèse sont également contrôlées plus localement par de multiples membres de la famille des récepteurs nucléaires : *Chicken Ovalbumin Upstream Promoter-Transcription Factor* (Coup-tf, Nr2f2), *Nerve Growth Factor-Induced clone B* (NgfiB ou Nur77, Nr4a1), Sf-1, Lrh-1, Shp et Dax-1 (336). Parmi ceux-ci, les récepteurs Sf-1 et Lrh-1 sont des régulateurs positifs de la synthèse des hormones stéroïdes gonadiques et surrénaliennes. Ils stimulent en effet l'expression de leurs gènes cibles communs Star, Cyp11a1, 3βHsd et Cyp17a1 codant les enzymes de la stéroïdogenèse (337)-(338) et (339)-(340).

Les récepteurs nucléaires orphelins Shp et Dax-1 sont quant-à eux des régulateurs négatifs de l'activité stéroïdogène des cellules de Leydig. Ce sont des récepteurs nucléaires particuliers dépourvus de domaine de liaison à l'ADN, mais ayant la capacité d'interagir avec d'autres membres de cette super-famille inhibant de ce fait leur activité transcriptionnelle. Les souris mâles $Shp^{-/-}$ présentent une augmentation de la synthèse testiculaire de testostérone indépendante de l'axe hypothalamo-hypophysaire (33). Les auteurs ont démontré que Shp réprime la stéroïdogenèse d'une part en inhibant l'expression de Sf-1 et Lrh-1, et d'autre part en interférant avec l'activation transcriptionnelle de Lrh-1 sur le promoteur des gènes Star, Cyp11a1 et 3βHsd. Contrairement au modèle d'invalidation de Shp, les souris $Dax\text{-}1^{-/-}$ ont un niveau d'expression des enzymes stéroïdogènes équivalent à celui des animaux sauvages, en dehors de l'aromatase dont l'expression est augmentée (341). Cependant, une étude récente a permis de montrer, *in vitro* et *in vivo*, que l'insuline réprime la stéroïdogenèse *via* l'induction de l'expression de Dax-1 (342), qui comme Shp est capable d'interagir avec Sf-1 et Lrh-1 (343)-(344).

Outre leur rôle dans le contrôle de l'expression des enzymes de la stéroïdogenèse, Sf-1 et Lrh-1 sont également impliqués dans la régulation du métabolisme du substrat de cette voie de biosynthèse : le cholestérol. Ils contrôlent en effet l'expression des gène codants le récepteur Sr-b1 qui fournit le cholestérol plasmatique aux tissus stéroïdogènes (345)-(346), et les enzymes *3-Hydroxy-3-Methylgutaryl-CoA* (Hmg-CoA) *synthase* et *reductase* impliquées dans la biosynthèse du cholestérol

(347)-(348). Ils participeraient ainsi à la régulation du transport et de la synthèse de novo de cholestérol dans les cellules de Leydig.

3.2.c. Autres facteurs régulateurs

Outre la Lh, la stéroïdogenèse est sous le contrôle d'autres facteurs hormonaux. Les hormones thyroïdiennes stimulent *in vitro* l'expression de Star et la production des stéroïdes sexuels par les cellules de Leydig (349). Les glucocorticoïdes exercent une action inhibitrice sur la stéroïdogenèse en réprimant l'expression des enzymes impliquées dans cette voie de biosynthèse et en induisant l'apoptose des cellules de Leydig, expliquant la diminution de la concentration de testostérone observée en situation de stress (350)-(351).

3.3. Métabolisme et mode d'action des androgènes

Une fois synthétisée et exportée des cellules de Leydig, la testostérone se trouve au contact des différents compartiments cellulaires du testicule sur lesquels elle pourra exercer son action. Une partie de la testostérone rejoint la circulation sanguine *via* les veines spermatiques afin d'atteindre ses tissus cibles en périphérie.

Dans le plasma, la testostérone n'est présente qu'en faible proportion sous forme libre (2%). Elle circule majoritairement sous forme liée, complexée aux protéines de transport TeBG (*Testosterone Binding Globulin*), SHBG (Sex Hormone Binding Globulin) ou à l'albumine (352). Dans ses tissus cibles, la testostérone peut agir directement ou indirectement par le biais de métabolites actifs aux actions totalement différentes, ce qui permet de renforcer et de diversifier ses effets biologiques.

La testostérone est métabolisée en 5α-dihydrotestostérone (Dht) sous l'action de la 5α-réductase, enzyme présente dans la plupart des organes androgéno-dépendants périphériques (épididyme, prostate, vésicule séminale, peau et foie) (353). La testostérone et la Dht agissent par l'intermédiaire d'un récepteur commun : le récepteur nucléaire Ar, dont le mode d'action a été décrit dans la partie ''classification des récepteurs nucléaires'' de ce manuscrit. Cependant, la Dht se fixe au récepteur Ar avec une affinité de liaison supérieure à celle de la testostérone, ce qui en fait un androgène aux activités hormonales plus puissantes que la testostérone (354). Chez l'Homme, la 5α-réductase est peu ou pas exprimée dans le testicule, la testostérone y est donc le seul androgène actif (355).

La testostérone peut également être métabolisée en un œstrogène puissant, l'œstradiol (E2). Cette réaction enzymatique est catalysée par l'aromatase, ou cytochrome P450arom, codée par le gène Cyp19. Le testicule assure 20% de la production totale d'œstrogènes, la majorité desquels étant issus de l'aromatisation périphérique (graisse, peau, rein, os , cerveau) (356). Les œstrogènes exercent leur

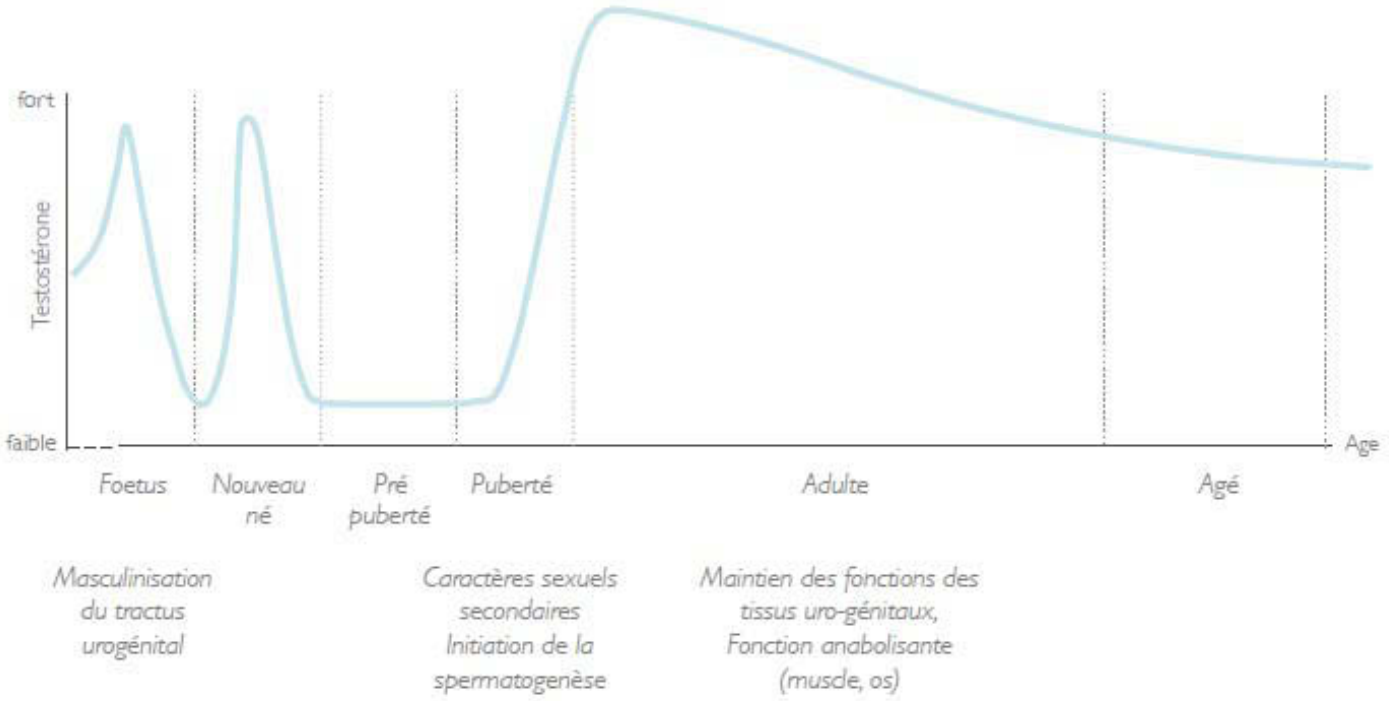

Figure 18 : Profil de sécrétion de la testostérone au cours de la vie d'un Homme et fonctions associées. Au cours du développement embryonnaire, les cellules de Leydig fœtales sont responsables d'un premier pic sécrétoire d'androgènes à l'origine de la masculinisation du tractus urogénital et de l'apparition des caractères sexuels primaires. Au moment de la puberté, l'augmentation de la pulsatilité de la GnRH est responsable d'un nouveau pic de synthèse des androgènes par les cellules de Leydig. Il permet le développement des caractères sexuels secondaires (apparition de la pilosité, augmentation de la masse musculaire, croissance osseuse) et l'initiation de la spermatogenèse. Chez l'adulte, le maintien des concentrations de testostérone est indispensable à l'entretien des cycles de spermatogenèse, ainsi qu'au maintien de la fonction des organes différenciés du tractus génital (prostate, épididyme, vésicule séminale). La testostérone possède également un pouvoir anabolisant favorisant le développement du muscle ou encore de l'os.

action au travers d'un récepteur nucléaire dont il existe deux isoformes α et β (*Estrogen Receptor*, Erα ou Nr3a1, et Erβ ou Nr3a2), et d'un récepteur membranaire couplé aux protéines G (Gper ou Gpr30). Leur mode d'action a été décrit dans la partie ''classification des récepteurs nucléaires'' de ce manuscrit. Bien que qualifiées d'hormones féminines, les œstrogènes jouent un rôle clé dans le maintien de la fertilité masculine, comme l'atteste le phénotype d'infertilité développé par des modèles animaux d'invalidation du gène codant l'aromatase ou le récepteur nucléaire aux œstrogènes de type α (357).

3.4. Rôles des androgènes

On distingue au cours de la vie d'un Homme trois fenêtres de temps particulièrement sensibles à la testostérone (**Figure 18**). Au cours du développement embryonnaire, les cellules de Leydig fœtales vont être responsables d'un premier pic sécrétoire d'androgènes à l'origine de la masculinisation du tractus génital et de l'apparition des caractères sexuels primaires (157). La testostérone va ainsi favoriser le développement du canal de Wolff en épididyme, canal déférent et vésicules séminales ; la Dht va quant-à elle entraîner la formation du pénis et du scrotum, et de la prostate à partir respectivement du tubercule et du sinus urogénital. La testostérone et la Dht agissent *via* leur récepteur nucléaire commun Ar. Chez l'Homme, des mutations affectant le gène codant le récepteur AR entraînent un syndrome de résistance aux androgènes (358). Celui-ci peut être partiel ou complet. Cette deuxième forme, la plus grave, se traduit alors par un tractus génital externe féminin, des testicules en position intra-abdominale ce qui est incompatible avec la spermatogenèse, et l'absence de développement de caractères sexuels secondaires féminins (aménorrhée, absence de pilosité axilaire et pubienne) au moment de la puberté.

Au moment de la puberté, les androgènes permettent le développement des caractères sexuels secondaires (apparition de la pilosité, augmentation de la masse musculaire, croissance osseuse), la mise en place de l'activité sécrétrice du tractus génital (épididyme, prostate, vésicules séminales) et l'initiation de la spermatogenèse.

Chez l'adulte, la testostérone est indispensable au maintien de la spermatogenèse. En effet, une diminution drastique de la production d'androgènes en réponse à une déficience en gonadotrophines, ainsi qu'une perte d'expression de leur récepteur Ar (souris ARKO) sont associés à une apoptose massive des cellules germinales et un arrêt de la spermatogenèse au stade de la première division de méiose (359)-(360)-(361). Par ailleurs, l'administration d'androgènes aux souris déficientes en gonadotrophines suffit à elle-seule à rétablir la spermatogenèse soulignant d'avantage le rôle essentiel des androgènes dans la spermatogenèse (360).

Ces données soulignent l'importance fondamentale des androgènes et de leur récepteur Ar pour maintenir la survie et la différenciation germinale. L'expression de Ar est largement répandue dans le testicule adulte. En effet, chez l'Homme et les rongeurs, il est exprimé dans les types cellulaires somatiques majeurs du testicule, à savoir les cellules de Leydig, de Sertoli et myoïdes péritubulaires. En revanche, son expression dans les cellules germinales reste à ce jour controversée. Certaines études ont localisé Ar dans les cellules germinales de plusieurs espèces, à des stades de différenciation bien spécifiques : spermatogonies et spermatocytes (362)-(363)-(364), ou spermatides (365). A l'inverse d'autres études n'ont détecté la présence de Ar dans aucun stade de la spermatogenèse (366)-(367)-(368)-(369). Ainsi, une question persistait-elle dans la communauté scientifique : le(s)quel(s) de ce(s) type(s) cellulaires(s) étai(en)t la cible des androgènes pour participer au contrôle de la spermatogenèse. L'obtention de souris dont le gène codant Ar a été invalidé spécifiquement dans les différents types cellulaires du testicule (germinal (G-AR$^{-/y}$) péritubulaire (PTM-ARKO), Leydig (L-AR$^{-/y}$) et Sertoli (SCARKO)) a permis de répondre à cette question.

3.4.a. Ar et cellules germinales

Les souris G-AR$^{-/y}$ ont été générées avec l'aide de la recombinase Cre, dont l'expression était sous le contrôle du promoteur du gène codant le marqueur méiotique Sycp1 exprimé dans les spermatocytes leptotène et zygotène. Ces animaux ne présentent aucune altération de la morphologie et de l'histologie du testicule, de la spermatogenèse, de la concentration plasmatique de testostérone ou encore de la fertilité, suggérant que l'expression de Ar dans les cellules germinales n'est pas indispensable à la spermatogenèse une fois la méiose initiée (370). De plus, la transplantation de spermatogonies provenant de souris ARKO dans les tubes séminifères de souris sauvages, conduit à une spermatogenèse qualitativement normale (371), soutenant d'avantage l'idée que si Ar est exprimé dans les cellules germinales, il n'est pas requis pour le maintien de leur survie et de leur différenciation.

3.4.b. Ar et cellules myoïdes péritubulaires

Les cellules myoïdes péritubulaires sont les seules à exprimer Ar dès la vie fœtale, et maintiennent son expression jusqu'à l'âge adulte (372)-(373). Les souris de 12 jours PTM-ARKO ont des testicules indiscernables de ceux des animaux sauvages d'un point de vue macroscopique et histologique. Ces données suggèrent que la signalisation androgénique médiée par les cellules péritubulaires n'est pas primordiale pour le développement fœtal et pré-pubère du testicule. Cependant, l'expression de Ar n'étant pas délétée dans toutes les cellules péritubulaires dans ce modèle, les cellules dans lesquelles l'expression de Ar persiste pourraient être suffisantes pour supporter le développement précoce du

testicule. En revanche, les souris adultes sont stériles et azoospermiques. Elles présentent en effet une diminution du poids des testicules dès l'âge de 15 jours et qui se maintien à l'âge adulte, associée à une réduction du nombre de cellules correspondant à tous les stades de la lignée germinale. L'action des androgènes *via* les cellules péritubulaires est donc indispensable au bon déroulement de la spermatogenèse (374). L'altération de ce processus pourrait s'expliquer en partie par un disfonctionnement des cellules de Sertoli comme l'atteste la diminution de l'expression de gènes sertolien androgéno-dépendants (Rhox5, Tubb3 et Eppin) et de leur activité sécrétrice reflétée par une réduction du diamètre de la lumière des tubes.

L'ablation de Ar dans les cellules péritubulaires se traduit de plus par un défaut de développement des cellules de Leydig (375), résultant probablement d'un défaut des signalisations *Insulin Growth Factor* (Igf), *Desert hedgehog* (Dhh), *Plateled-Derived Growth Factor* (Pdgf) impliquées dans la différenciation des cellules de Leydig adultes (376)-(377)-(378). Les souris PTM-ARKO présentent en effet à l'âge adulte une élévation de la concentration intra-testiculaire de testostérone par rapport à celle des souris témoins, alors que la concentration plasmatique de cet androgène n'est pas altérée, suggérant un défaut de fonctionnement des cellules de Leydig, en particulier une altération des systèmes de transport impliqués dans l'export de la testostérone dans les vaisseaux sanguins (374). Il est intéressant de noter que toutes les cellules de Leydig ne sont pas atteintes de la même façon. Deux populations de cellules de Leydig sont en effet présentes en proportion égale dans le tissu interstitiel : une population immature, et une population mature qui devra fonctionner à plein régime afin de compenser le disfonctionnement des cellules immatures.

3.4.c. Ar et cellules de Leydig

Les androgènes exercent une action autocrine sur les cellules de Leydig *via* leur récepteur Ar afin de contrôler leur propre synthèse. Les souris L-AR$^{-/y}$ présentent en effet une diminution des concentrations plasmatiques de testostérone malgré des taux élevés de Lh. Cette hypo-androgénie s'explique par un défaut d'expression de gènes codant les enzymes clées de la stéroïdogenèse Cyp17a1, 3βhsd et 17βhsd, et est associée à un arrêt de la spermatogenèse au stade de spermatide ronde à l'origine d'une stérilité (370)-(379).

3.4.d. Ar et cellules de Sertoli

Les souris SCARKO sont stériles. Aucun spermatozoïde n'est détecté dans l'épidydime. Le poids de leurs testicules est significativement diminué du fait d'une déplétion en cellules germinales dûe à une spermatogenèse incomplète : on observe en effet un blocage de la spermatogenèse au stade de première division de méiose (spermatocyte primaire) prévenant ainsi la formation des spermatides

rondes. Cette diminution du nombre de cellules germinales est associée à une augmentation de l'apoptose et à un défaut des fonctions sertoliennes dont le nombre n'est pas altéré (318)-(319)-(320). La présence d'un récepteur Ar fonctionnel dans les cellules de Sertoli semble en effet indispensable à la mise en place et au maintien de l'intégrité de la BHT, comme l'atteste la diminution de l'expression de gènes codant plusieurs protéines de jonctions inter-cellulaires (Claudine 3, Claudine 11, Occludine, Gelsoline, Cadhérine 2, Jam 3 ou ZO-1) chez les animaux SCARKO (380)-(321)-(238). Les cellules de Sertoli montrent par ailleurs un défaut de maturation nucléaire et de polarisation (positionnement abberrant du noyau et de composants du cytosquelette) en lien avec la dérégulation des gènes codant pour la vimentine et la laminine α5, protéines impliquées respectivement dans la composition du cytosquelette et de la matrice extra-cellulaire (321)-(238). Enfin les fonctions sécrétrices et nutritives des cellules de Sertoli vis-à-vis des cellules germinales semblent être altérées au vu de la diminution de l'expression de gènes codant des protéines de transport (Abp, *transferrin*, *epidermal Fatty-Acid-Binding Protein* (eFABP)) et facteurs paracrines (*Cyclin A1* , *Sperm-1*) nécessaires à la différenciation des cellules germinlaes (318)-(321). Ces résultats démontrent que la signalisation androgénique dans les cellules de Sertoli est primordiale au maintien de la spermatogenèse *via* le contrôle des fonctions sertoliennes (mise en place et maintien de la BHT, maintien de la structure et la morphologie des cellules de Sertoli affectant l'intégrité des tubes séminifères).

Comme mentionné dans la première partie de ce manuscrit, la testostérone régule également d'autres fonctions sertoliennes par le biais de mécanimes moléculaires non génomiques, caractérisés par l'activation de voies de signalisations cytoplasmiques. Une population de récepteurs Ar est ainsi localisée à proximité de la membrane plasmique des cellules de Sertoli. Suite à leur stimulation par la testostérone, ces récepteurs sont capables d'intéragir et d'activer la protéine kinase Src, déclenchant une cascade de phosphorylations impliquant le récepteur du facteur de croissance Egf, et la voie des Mapk, aboutissant à l'activation du facteur de transcription Creb (7). La mise en jeu de cette voie de signalisation permet aux stéroïdes d'étendre la portée de leur action, en modulant l'expression d'un panel de gènes bien plus large que celui sous l'influence de leur seule action génomique. La protéine Src permet notamment de moduler le statut de phosphorylations des protéines cadhérines et caténines impliquées dans la formation des jonctions adhérentes, et par la même la force de leur interaction (381)-(382). C'est également par l'intermédiaire de ces kinases que la testostérone permet le maintien de l'adhésion des spermatides aux cellules de Sertoli, et le relargage des spermatozoïdes dans la lumière des tubes séminifères au moment de la spermiation (383)-(320).

Partie 3 : Objectifs du travail de thèse.

Fxrα est le récepteur nucléaire des acides biliaires, exprimé majoritairement dans le foie, l'intestin, les reins et les glandes surrénales. L'intérêt pour ce dernier est devenu croissant au cours des dernières années, de part le rôle central qu'il joue dans le contrôle de l'homéostasie du cholestérol, des acides biliaires, des triglycérides ou encore du glucose, le définissant ainsi comme une cible thérapeutique de choix pour le traitement de pathologies métaboliques telles que la dyslipidémie ou le diabète.

Plus récemment, plusieurs études indépendantes ont localisé Fxrα dans le testicule, et plus précisément dans les cellules de Leydig. Les récents travaux de l'équipe ont par ailleurs mis en évidence la présence de ses ligands, les acides biliaires, dans cet organe en condition physiologique. De plus, Fxrα est également activable par l'androsterone, un intermédiaire de la synthèse des stéroïdes sexuels, produit par les cellules de Leydig. L'ensemble de ces données soulèvent la question du rôle potentiel de Fxrα dans le testicule, mais les études menées à ce sujet restent jusqu'à présent peu nombreuses, et focalisées sur son implication dans le contrôle du métabolisme des stéroïdes. L'activation *in vivo* de Fxrα par un agoniste synthétique conduit ainsi chez l'adulte à une répression de la stéroïdogenèse (33). Des études *in vitro* menées dans une lignée tumorale de cellules de Leydig de rat ont par ailleurs démontré sa capacité à inhiber la synthèse d'oestrogènes via la répression de la transcription du gène codant l'aromatase (38).

Outre son rôle dans le contrôle de l'activité endocrine des cellules de Leydig, l'impact de l'activation *in vivo* de Fxrα sur la physiologie plus globale du testicule n'a jamais été abordé à ce jour. De telles études seraient pourtant pertinentes étant donné que plusieurs molécules agonistes ou antagonistes synthétiques de Fxrα ont été développées pour le traitement de pathologies métaboliques telles que la dyslipidémie ou le diabète, certaines faisant l'objet de test clinique.

Dans ce contexte, l'objectif de ce travail de thèse était d'étudier le rôle de Fxrα dans la physiologie et la patho-physiologie du testicule. Il s'est articulé autour de trois projets visant à établir : **1) l'implication potentielle de Fxrα dans les altérations de la physiologie du testicule observée en condition pathologique, et plus précisément dans un contexte de cholestase ; 2) les conséquences de l'activation de la signalisation associée à Fxrα au moment de la mise en place des fonctions de reproduction ; 3) le rôle plus global exercé par Fxrα dans la physiologie du testicule.**

Les résultats présentés ci-après ont été obtenus grâce à l'analyse phénotypique, biochimique, histologique et moléculaire d'un modèle murin dont le gène codant Fxrα a été invalidé. De plus, l'utilisation de culture primaire de cellules de Leydig, ainsi que de lignées cellulaires (MA10 : Leydig,

GC1-spg : germinales, 42GPA9 : Sertoli) nous ont permis de préciser sur le plan mécanistique et moléculaire les conclusions obtenues *in vivo*.

RESULTATS

Article I

Bile acids impact urogenital tract* via *PXR signaling pathways in FXRα deficient mice.

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En préparation

Les acides biliaires sont des dérivés du cholestérol impliqués dans la solubilisation et la digestion des graisses alimentaires. L'intérêt pour ces derniers n'a cessé de croître ces dernières années, depuis leur identification en tant que véritables molécules de signalisation. Plusieurs récepteurs aux acides biliaires ont dès lors été identifiés, les deux principaux étant le récepteur membranaire Tgr5, et le récepteur nucléaire Fxrα.

La concentration plasmatique d'acides biliaires est très précocement augmentée en cas de pathologies hépatiques, et a été récemment démontrée par l'équipe (Baptissart *et al.* 2014) comme étant responsable de la survenue de troubles de la fertilité masculine (384). D'un point de vue moléculaire, l'effet délétère des acides biliaires sur la physiologie testiculaire est dépendant du récepteur membranaire Tgr5, et se traduit par une rupture de la barrière hémato-testiculaire associée à une nette augmentation de l'apoptose des cellules germinales post-méiotiques.

Bien que ces travaux (384) aient montré que Tgr5 est le médiateur principal de l'effet délétère des acides biliaires sur la physiologie testiculaire (analyse des souris $Tgr5^{-/-}$), la question de l'implication potentielle du récepteur Fxrα dans ce processus de physiopathologie testiculaire demeurait. Plusieurs arguments venaient étayer ce questionnement : 1- le récepteur Fxrα est présent dans les cellules de Leydig, dans lesquelles son activation conduit à une répression de la stéroïdogenèse ; 2- l'expression de certaines enzymes stéroïdogènes semble diminuée en réponse aux acides biliaires (Star, Cyp11a1, Cyp17a1, données non publiées), suggérant une activation de la voie de signalisation associée à Fxrα en réponse à l'exposition à un tel régime. Nous avons donc décidé de soumettre à des animaux $Fxr\alpha^{-/-}$ un régime supplémenté en acides biliaires (régime CA).

Bile acids impact urogenital tract *via* PXR signaling pathways in FXRα deficient mice.

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Abstract.

Bile acids have recently been demonstrated as molecules with endocrine activities controlling several physiological functions such as immunity and glucose homeostases. They act mainly through two receptors, the nuclear receptor Farnesoid-X-Receptor alpha (FXRα) and the G-protein coupled receptor (TGR5). Interestingly, it has been described that FXRα is expressed in the sterodogenic tissues, where it seems to control steroid production. In addition, FXRα also participates in steroid catabolism in the liver and interferes with the steroid signaling pathways in target tissues via crosstalk with steroid receptors. Although FXRα is expressed within the testes, the potential effect of BAs on testis physiology and male fertility has not been explored so far. Here, we demonstrate that mice invalidated for the gene encoding FXRα fed a diet supplemented with cholic acid have reduced fertility subsequent to testicular defects. In this context, BA exposure mainly affects germ cell survival. The present study suggests that in the absence of FXRα, BA must act through PXR signaling pathways to mediate deleterious effects on testicular physiology.

Introduction

Bile acids (BAs) are cholesterol metabolites that have been extensively studied in recent decades. BAs have recently been described as signaling molecules involved in many physiological functions, such as glucose and energy metabolisms. These signaling pathways mainly involve the activation of two BA receptors, the nuclear receptor farnesoid X receptor (FXRα ; NR1H4) or the BA-G protein-coupled receptor-1 (GPBAR-1; TGR5). BA levels are increasedduring liver diseases (1), (2) and have been defined as the most consistent change in the early phase of several liver diseases out of 1900 metabolites screened in the plasma, urine and liver (3).

We recently demonstrate that BA elevation impacts testicular pathophysiology and fertility (4). The major functions of the testis include production of male gametes and secretion of testosterone. After 4 months, in wild-type males, BAs exposure leads to a decrease of spermatozoa production by the testis. These effects are mediated by TGR5 within the germ cell lineage. There TGR5 activation leads to alterations of cell-cell interactions and blood-testis-barrier rupture which in turn lead to apoptosis of post-meiotic germ cells. These data showed that TGR5 activation does not impact testicular testosterone homeostasis. In contrast, some

roles of FXRα on endocrine testicular function were previously suggested using *in vitro* approaches with impact on estrogen metabolism associated with the reduction of the aromatase gene expression (5). This is mainly due to competition with steroidogenic factor-1, a known inducer of steroidogenesis. In the same line of evidence, testosterone synthesis is altered, *in vivo*, in short term exposure to the synthetic agonist of FXRα (e.g. GW4064) (6). The negative effects of FXRα signaling pathways are mediated by the increased expression of the Small Heterodimer Partner (SHP, NR0B2). SHP inhibits the expression of steroidogenic genes, on the one hand by inhibiting the expression of the nuclear receptors steroidogenic factor-1 (SF-1; NR5A1) and liver receptor homolog-1 (LRH-1; NR5A2), and on the other hand by directly repressing the transcriptional activity of LRH-1.

If BAs exposure is known to alter male fertility and that FXRα is a major regulator of BAs homeostasis, its exact roles in the links between liver disorders and testis physiopathology still need to be defined.

Using both genetic and pharmacologic strategies in mouse, we show here that FXRα controls hepatic catabolism of testosterone. In addition, in the context of FXRα deficiency, BA exposure results in a strong alteration of testis physiology, within only 1 month, with a decrease of sperm productionin a TGR5 independent manner. Elevated plasma BA levels in $Fxr\alpha^{-/-}$ mice led to germ cell apoptosis associated with altered expression of specific meiotic genes. In addition, our results suggest that in $Fxr\alpha^{-/-}$ mice bile acids could signal through Pregnane-X-Receptor (PXR; NR1I2).

Material & Methods

Ethics Statement. This study was conducted in accordance with the current regulations and standards approved by the Animal Care Committee (CEMEA Auvergne; protocol CE 07-12).

Animals. C57Bl/6J were purchased from Charles River Laboratories (c) (L'Arbresle, France), $Fxr\alpha^{-/-}$ mice used have been previously described (4), (7). Mice used in this study were maintained on housed in temperature-controlled rooms with 12 hours light/dark cycles. Mice had *ad libitum* access to food and water. Nine-week- old mice were fed to D04 diet (control) or D04 diet supplemented with 0.2% cholic acid (CA-diet) (SAFE, Augy, France) for 30 days. As $Fxr\alpha^{-/-}$ mice are quite sensitive to CA-diet, they were fed 5 days with CA-diet and 2 following days with the control diet. This sequence was repeated until sacrifice.

Histology. After diet exposure, the testes were collected, formalin-fixed and embedded in paraffin, and 5 µm-thick sections were prepared and stained with hematoxylin/eosin (n=6-10 animals per group).

For the analysis of the blood-testis barrier integrity, 15 µl of EZ-Link Sulfo-NHS-LC-Biotin (7.5 mg/ml) was injected intra-peritoneally (200µl) or an intratesticular (15µl) injection of 0.6 mg of cholyl-lysyl-fluorescein (BD Bioscience, Le Pont de Claix, France) (4). Thirty min after injection, the testes were harvested, formalin-fixed and embedded in paraffin, and 5 µm-thick sections were prepared.

TUNEL analysis. TUNEL experiments were performed as previously described (8) on 5 µm of testis fixed in PFA 4%. In each testis, at least 100 random seminiferous tubules were counted. The results are expressed as the number of tubules with either spermatocytes or spermatids TUNEL-positive per 100 seminiferous tubules.

Endocrine Investigations. Testosterone was extracted from testis as previously described (8). Intra-testicular and plasma testosterone levels were measured using a commercial kit (Diagnostic Biochem, London, Canada).

Bile acid measurements. The BA measurements were performed as previously described (4) and using ELISA assays as recommended by manufacturer (Crystal Chem, Inc. Cat# 80470).

Real-Time RT-PCR. RNA from testis samples was isolated using Nucleospin RNA L (Macherey-nagel, Hoerdt, France). cDNA was synthesized from total RNA with the MMLV reverse transcriptase and random hexamer primers (Promega, Charbonnière Les Bains, France). The real-time PCR measurement of individual cDNAs was performed using SYBR green dye (Master mix Plus for SYBR Assay, Eurogentec, Angers, France) to measure duplex DNA formation with the EppendorfRealplex system. The sequences of primers are reported in 4, 6, 8–11. Standard curves were generated with pools of testis cDNA from animals with different genotypes and/or treatments. The results were analyzed using the ΔΔct method.

Results/Discussion

Fxr$\alpha^{-/-}$ males are hypersensitive to bile acid diet. Consistent with previous studies (12), Fxr$\alpha^{-/-}$ mice were highly sensitive to diet supplemented with cholic acid (CA-diet). They showed important body weight loss which was associated with drastic alteration of testicular histology **(Suppl 1**). In order to be in an ethical context, we used protocol of alternating diets (cycles of normal diet and 0.2% CA diet, see method section). This led to a smaller body weight

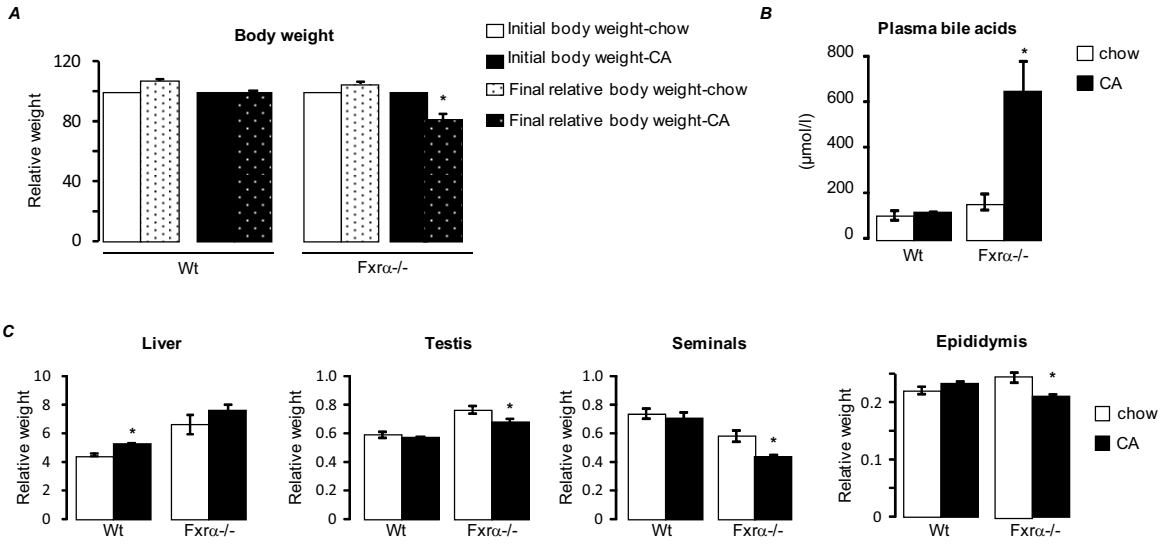

Figure-1: A/ Relative body weights throughout the experimental procedure in wild-type and Fxr$\alpha^{-/-}$ mice fed either chow or diet supplemented with 0.2% cholic acid for 1 month . Initial body weight was arbitrarily set as 100%. **B/** Plasma bile acid levels in wild-type and Fxr$\alpha^{-/-}$ mice fed a control or CA diet for 1 month. (n = 19–25 per group). **C/** Relative liver, testis, seminals and epididymis weights normalized to body weight in wild-type and Fxr$\alpha^{-/-}$ mice fed a control or CA diet for 1 month. Data represent mean ± SEM; Statistical analyses: * p<0,05.

decrease (less than 20%) **(Fig. 1A)** which was comparable to the one observed, in previous study, for wild-type males fed 0.5% CA diet (4). It is interesting to note that in this alternation of diets, no impact was observed on body weight of wild-type mice **(Fig. 1A)**. Due to the alternation of diets, plasma BA levels were not altered in wild type males, whereas a high increase (4-fold) was found in Fxr$\alpha^{-/-}$ mice**(Fig. 1B)**. This is consistent with the major role of FXRα in the control of BA homeostasis (12). BA exposure is associated with a small increase of liver weight in wild-type mice. In contrast, whereas FXRα deficiency led to increase of liver weight no additional effect of of BA-diet was observed in Fxr$\alpha^{-/-}$ **(Fig. 1C)**. Regarding the urogenital tract, BA exposure has no impact in wild-type males, whereas it led to lower weights of seminals, epididymis and testis **(Fig. 1C)** in Fxr$\alpha^{-/-}$ males.

FXRα controls liver injury and testosterone catabolism. Testis, seminals and epididymis are three organs dependent of androgen status. Measurements of plasma testosterone concentrations revealed decreased levels in wild-type mice exposed to BA-diet compare to control-diet group **(Fig. 2A)**. This is consistent with previous published data which showed that this effect of BA-diet on plasma testosterone is

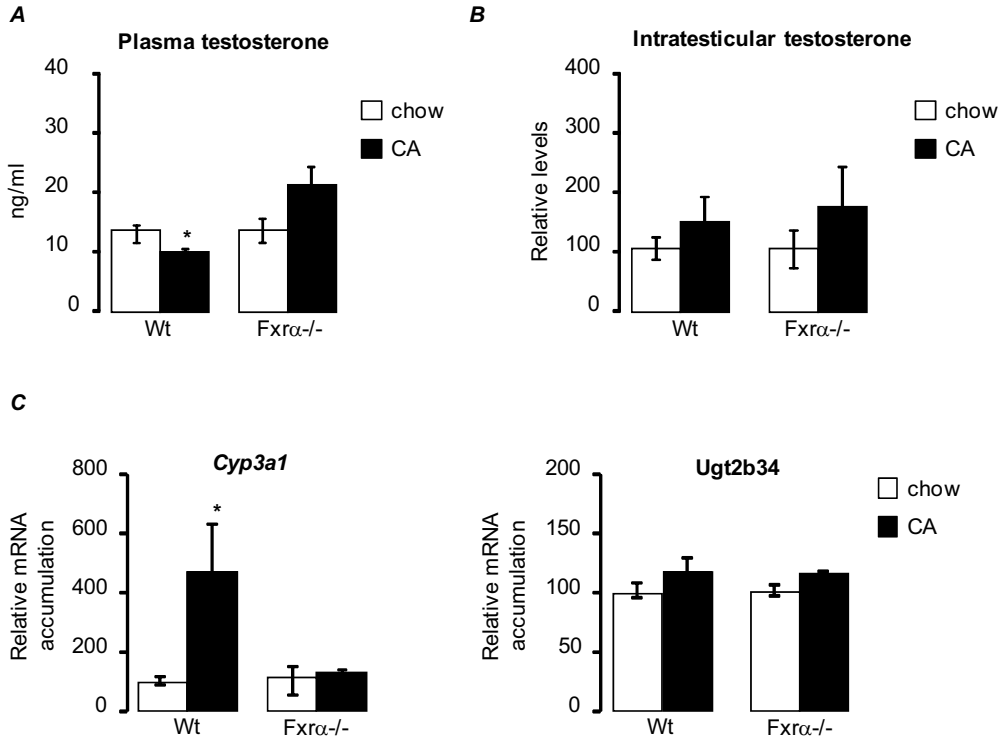

Figure-2: A/ Plasma testosterone levels in wild-type and Fxr$\alpha^{-/-}$ mice fed a control or CA diet for 1 month. **B/** Relative intratesticular testosterone levels in *wild-type* and Fxr$\alpha^{-/-}$ mice fed a control or CA diet for 1 month. **C/** Hepatic mRNA expression of Cyp3a11, Ugt2b34 and *Igf1* normalized to *β-actin* mRNA levels in wild-type and Fxr$\alpha^{-/-}$ mice fed a control or CA diet for 1 month (n = 6 per group). In all panels, control diet groups were arbitrarily fixed at 100%. In all panels, data are expressed as means ± standard error of the mean. Statistical analysis: $*P < 0.05$.

independent of TGR5 activation (4). In the previous work from Baptissart *et al*., it was suggested that the lower plasma testosterone levels are not due to altered testosterone production by the testis. The present study supports this idea as the analyses demonstrated no difference on the intra-testicular testosterone levels between groups **(Fig. 2B)**. These results were sustained by the lack of effect of BA-diet on the mRNA accumulation of androgeno-dependent genes in both wild-type and Fxr$\alpha^{-/-}$ males (*Pem*, *osp*) **(data not shown)**. In the previous study, it was hypothesized that BA-exposure might lead to hepatic catabolism of testosterone leading to decrease plasma levels. Here we identify the main role of FXRα in this process as no effect of BA-exposure was observed in Fxr$\alpha^{-/-}$ males regarding plasma testosterone levels **(Fig. 2A)**. This data is consistent with the major role of FXRα within liver physiology. In that line we showed that in wild-type males, BA-diet induced the mRNA accumulation of genes involved in such mechanism such as *Cyp3a11* **(Fig. 2C)** whereas no effect was

observed in Fxr$\alpha^{-/-}$ males. No effect of BA exposure was observed on *Ugt2b34* mRNA accumulation **(Fig. 2C)**.

Even though it is of interest to define FXRα as major actor of hepatic metabolism of steroids, this is not consistent with the observed effects of BA-exposure on male genital tract in Fxr$\alpha^{-/-}$. We thus decided to focus our study on one of the altered organ : the testis.

Dietary BA supplementation alters testicular physiology. The decreased testis weight in Fxr$\alpha^{-/-}$ mice exposed to BA-diet, suggests abnormalities in spermatozoa production.

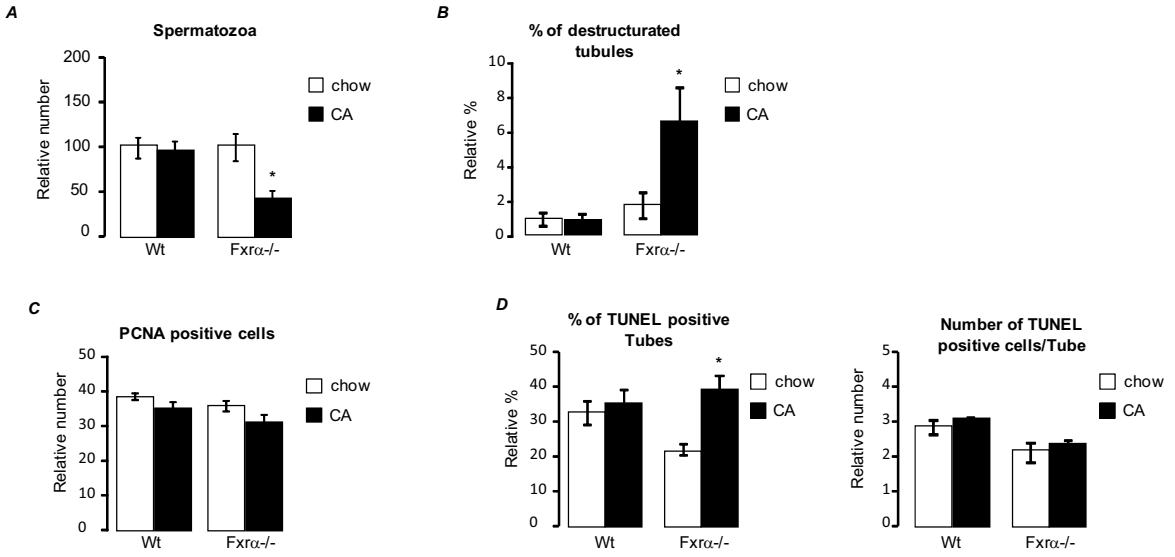

Figure-3: A/ Spermatozoa count in the heads of the epididymis of wild-type and Fxr$\alpha^{-/-}$ males exposed to 1 month of control or CA diets (n = 10-20 per group). Control diet group was arbitrarily fixed at 100%. **B/** Quantification of the number of completely destructured tubules per 100 seminiferous tubules in wild-type and Fxr$\alpha^{-/-}$ after 1 month of control or CA diets (n = 10-20 per group). **C/** The number of PCNA-positive cells is indicated as the number of positive cells per 100 seminiferous tubules (n = 10-20). Control-diet–treated mice were arbitrarily fixed at 100%. **D/** Apoptosis in wild-type and Fxr$\alpha^{-/-}$ mice exposed to control or CA diets (n = 10-20 per group) analyzed by TUNEL staining. Quantification of TUNEL analyses. The number of TUNEL-positive spermatocytes is indicated as the number of positive cells per 100 seminiferous tubules; the number of tubules with TUNEL-positive cells is indicated as the number of positive tubes per 100 seminiferous tubules (n = 10-20). Control-diet–treated mice were arbitrarily fixed at 100%.

In all of the panels, data are expressed as the means ± standard error of the mean. Statistical analysis: $*P < 0.05$.

This was sustained by a diminished sperm count by 50% in Fxr$\alpha^{-/-}$ mice following BA-exposure compare to chow-diet Fxr$\alpha^{-/-}$ mice **(Fig. 3A)**. Together these data suggest abnormal germ cell production within the testis. Consistently, a higher

number of destructurated seminiferous tubules was observed in Fxr$\alpha^{-/-}$ mice exposed to BA-diet compare to control-diet group **(Fig. 3B)**. Note that no effect of BA-diet was found in wild-type males regarding sperm count **(Fig. 3A).** Moreover, testicular histology of wild-type males was not affected **(Fig. 3B)**. Altered germ cell differentiation in response to BA-diet in Fxr$\alpha^{-/-}$ males was not due to differences in proliferation process **(Fig. 3C)**. Interestingly germ cell showed a higher apoptotic rate in Fxr$\alpha^{-/-}$ males compare to control groups **(Fig. 3D)**. The apoptotic process affected pre-meiotic or meiotic germ cells. Again wild-type males were not affected **(Fig. 3D)**.

The effects are not TGR5 dependent. The lack of FXRα suggests that BA must act through other signaling pathways. If previous study defined the major role of TGR5 in the pathophysiology of the testis; several elements of the present study suggest that the observed effects in Fxr$\alpha^{-/-}$ males in response to BA-diet are TGR5 independent.

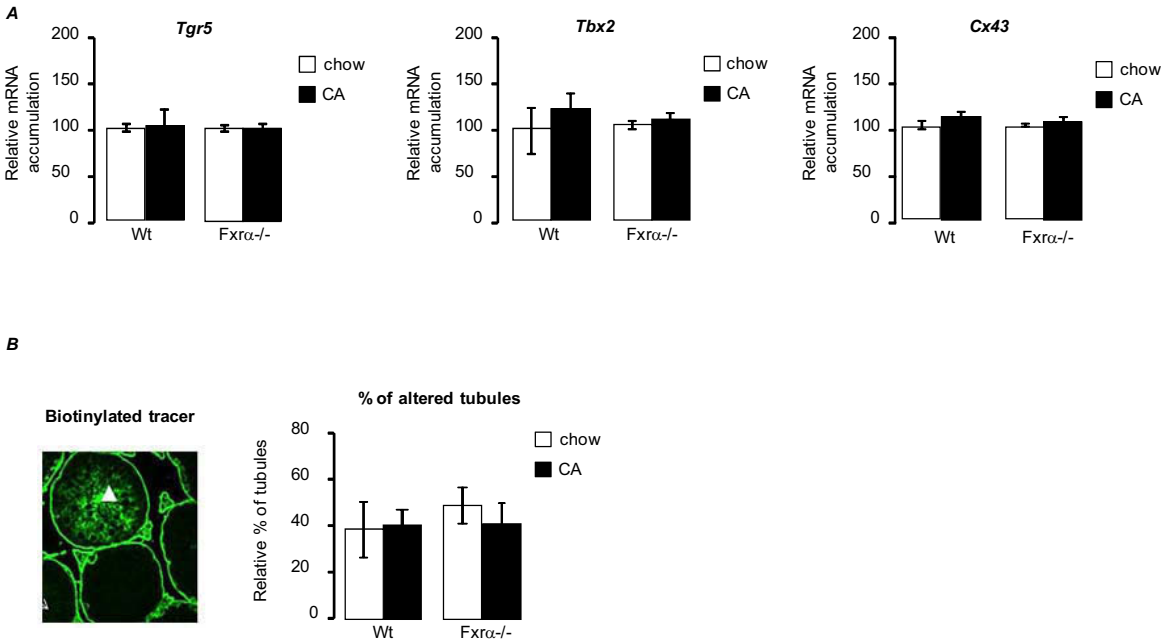

Figure-4: A/ Testicular mRNA expression of Tgr5, Tbx2 and *Cx43* normalized to *β-actin* mRNA levels in wild-type and Fxr$\alpha^{-/-}$ mice fed a control or CA diet for 1 month (n = 6 per group). **B/** BTB integrity, as measured by the stained testes for EZ-link biotinylated. Representative micrographs of mice fed 1 month with a control diet or CA diet (n = 10-15 per group). Arrow indicates a tubule with a slight infiltration of EZ-link biotinylated; arrowhead indicates a tubule with a high intensity of infiltration. The original magnification was 100×. Quantification of the number of tubules with infiltration per 100 seminiferous tubules after 1 month of a control or CA diet (n = 10-15 per group).

In all panels, control diet groups were arbitrarily fixed at 100%. In all panels, data are expressed as means ± standard error of the mean. Statistical analysis: $*P < 0.05$.

Indeed, we previously demonstrate that in wild-type males, long term exposure to BA-diet (2 to 4 months) leads to alteration of the expression of genes involved in cell-cell interactions and in turn induces rupture of the BTB and then apoptosis of spermatids (post-meiotic cells). Here the apoptotic germ cells are pre-meiotic and/or meiotic germ cells and not post-meiotic ones. In addition, results showed no alteration of *Tgr5*, *Tbx2* and*Cx43* mRNA accumulations in wild-type and Fxr$\alpha^{-/-}$ males in response to CA-diet **(Fig. 4A)**. This lack of effects on TGR5 pathway was supported by the fact that BTB was not altered in Fxr$\alpha^{-/-}$ males exposed to BA-diet **(Fig. 4B)**. These differences must be due to the fact that in contrast to previous study by Baptissart *et al.*, we have used alternation of diets due to the hyper-sensibility to BA-diet of *Fxrα* invalidated mice

Germ cell apoptosis is correlated with altered meiotic gene expression. It has been previously demonstrated that germ cell survival is under the control of androgen homeostasis (8). However, as mentioned above, intra-testicular levels of testosterone were not affected in Fxr$\alpha^{-/-}$ exposed to BA-diet compare to control group. We thus decided to explore other possibilities. As apoptosis occurred in pre-meiotic and/or meiotic steps, we first analyze the mRNA accumulation of genes involved in spermatogenesis. Here we demonstrate that in the context of *Fxrα* deficiency, BA-diet do not alter genes expressed in early spermatogenesis step as no effect was observed for gene specific of pre-meiotic spermatogonia such as *Plzf*, **(Fig. 5)**. In contrast, BA exposure specifically induced a decreased mRNA accumulation of two key genes of the meiotic process: the *Stimulated by retinoic acid gene-8* (*Stra-8*) and *DNA meiotic recombinase-1* (*Dmc-1*) **(Fig. 5)**; whereas others were not altered such *G9a, Sycp3* and *Ccna1*. This suggests a specific signaling mechanism. These results support that BA-diet altered the entry and/or the progress into meiosis. In that line the mRNA accumulation of post-meiotic gene such as *Tpn-1* (Transition protein-1) was decreased only in Fxr$\alpha^{-/-}$ males following BA exposure **(Fig. 5)**.

Interestingly, a previously link has been made between the small heterodimer partner (SHP), a known target gene of FXRα and the meiotic process (9), as SHP inhibit germ cells differentiation through the inhibition of *Stra8* expression. In that line, we show here that in the Fxr$\alpha^{-/-}$ testis, *Shp* mRNA accumulation was increased following BA-exposure **(Fig. 5)**. In contrast no effect on *shp* was observed in wild-type males **(Fig. 5)**.

No effect was seen on *Rarα* and *Cyp26a1* following CA-diet either neither in wild-type nor Fxr$\alpha^{-/-}$ males **(Fig. 5)**.

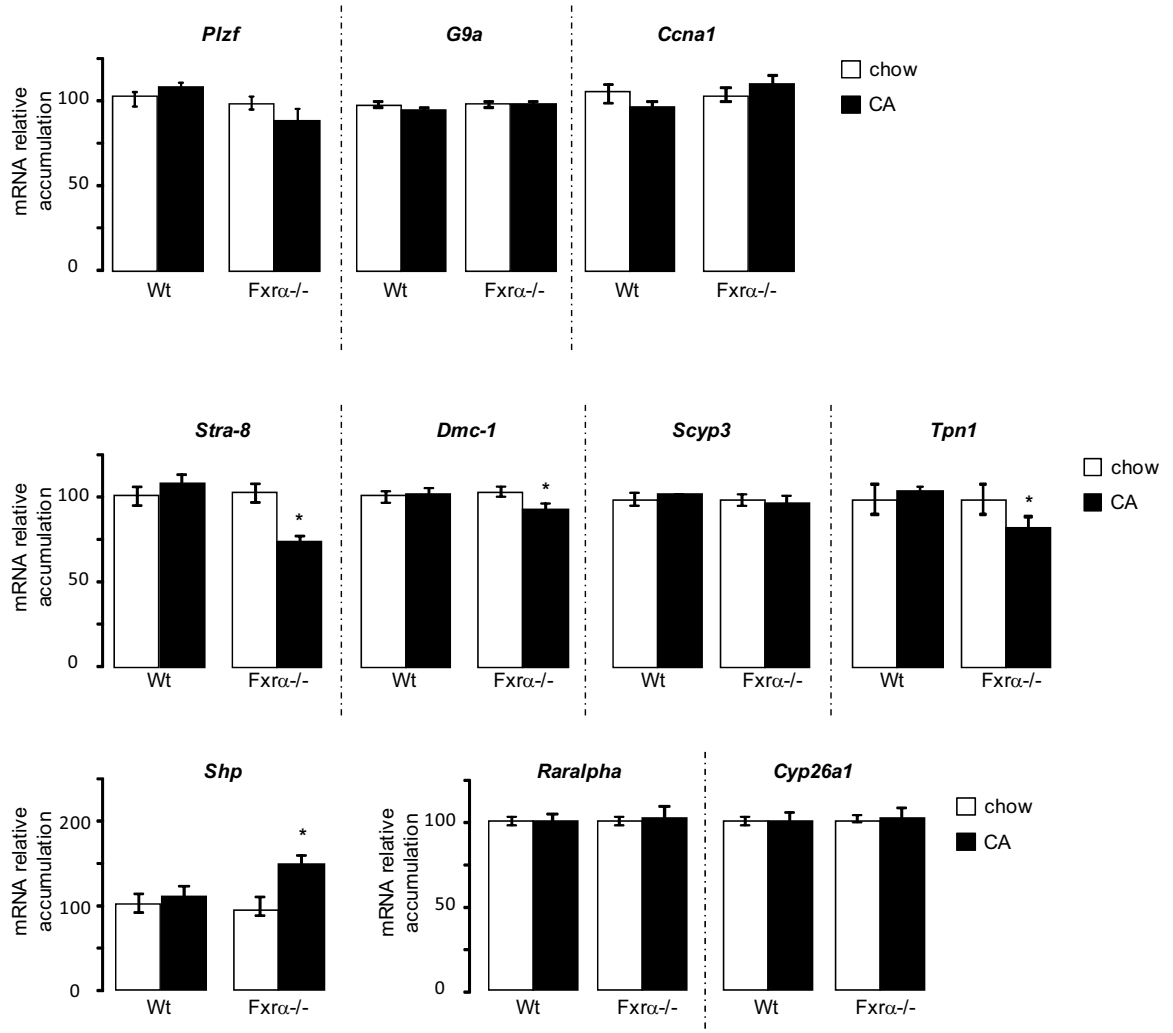

Figure-5: Testicular mRNA expression of *Plzf*, *G9a*, *Ccna1*, *Stra-8*, *Dmc-1*, *Scyp3*, *Tpn1*, *Shp*, *Raralpha* and *Cyp26a1* normalized to *β-actin* mRNA levels in wild-type and Fxr$\alpha^{-/-}$ mice fed a control or CA diet for 1 month (n = 6 per group).

In all panels, control diet groups were arbitrarily fixed at 100%. In all panels, data are expressed as means ± standard error of the mean. Statistical analysis: $*P < 0.05$.

Potential involvement of PXR. In order to understand how SHP is regulated in Fxr$\alpha^{-/-}$ mice exposed to BA-diet, we decided to explore the potential role of PXR as *Shp* was defined as a PXR target gene (13), (14). No effect on *Pxr* expression was observed following BA-exposure in either wild-type or Fxr$\alpha^{-/-}$ males **(Fig. 6A).** As BAs have been defined as potential ligand of PXR (15), we hypothesized that in the absence of FXRα, BA will be able to activate PXR and in turn its target genes.

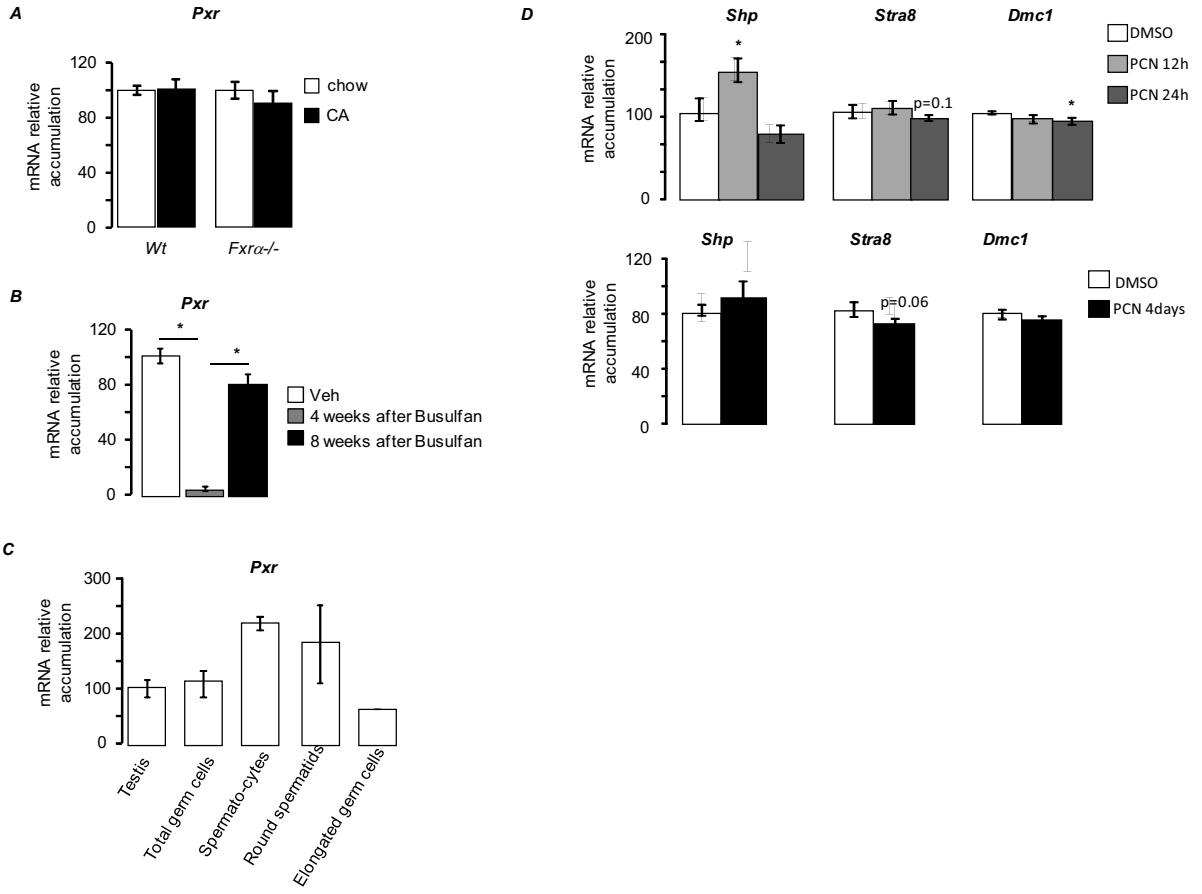

Figure-6: A/ Testicular mRNA expression of Pxr normalized to *β-actin* mRNA levels in wild-type and Fxrα$^{-/-}$ mice fed a control or CA diet for 1 month. **B/** Testicular mRNA accumulation of *Pxr* normalized to *β-actin* mRNA levels in whole testes of C57BL/6J mice treated with busulfan (20 mg/kg, one injection IP) at vehicule or busulfan 4, or 8 weeks (n = 8 per group). Data are expressed as means ± standard error of the mean. **C/** mRNA expression of *Pxr* normalized to *β-actin* mRNA levels in all testis or purified germ cells, spermatocytes, round spermatids or elongated germ cells. **D/** Testicular mRNA expression of Shp, *Stra-8*, *Dmc-1* normalized to *β-actin* mRNA levels in wild-type mice treated for 12hrs, 24 hrs or 4 days with the PXR agonist PCN (n = 5 per group). In all panels, control diet groups were arbitrarily fixed at 100%. In all panels, data are expressed as means ± standard error of the mean. Statistical analysis: $*P < 0.05$.

These results suggest that differentialactivation of PXR between wild-type and Fxrα$^{-/-}$ males could explain the observed differential regulation of *Shp* by BAs in the two genotypes.

Few are known about PXR in the testis. We first established in which testicular cell types express PXR. Using classical approach with busulfan (4), results show that PXR is mainly expressed in germ cell lineage **(Fig. 6B)**. Purification of specific steps of germ cells allows us to define the expression of PXR within pachytene spermatocytes and round spermatids **(Fig. 6C)**. To validate the impact of PXR within the testis, we exposed C57Bl6 mice to the

PXR agonist PCN during 12hours, 24 hours or 4 day. The efficiency of the PCN treatment was supported by the increase of liver weight relative to body weight **(data not shown)**. These experiments demonstrate that the PXR signaling pathway is active within the testis as PCN treatment led to the increase of *Shp* mRNA accumulation at 12 hours and then decrease after 24-hours **(Fig. 6D)**. This is consistent with the classical regulation of SHP which controls its own expression (16). Here the analysis of meiotic genes showed that 24 hours after PCN exposure, the mRNA accumulation of *Dmc1* was decreased **(Fig. 6D)**. Then after 4 days of PCN treatment, the mRNA expression of *Stra8* was decreased **(Fig. 6D)**. These results are consistent with known downstream signaling pathway of SHP (9) and suggest that in Fxr$\alpha^{-/-}$ fed CA-diet, PXR signaling might *via* the regulation of *Shp* leads to germ cell apoptosis.

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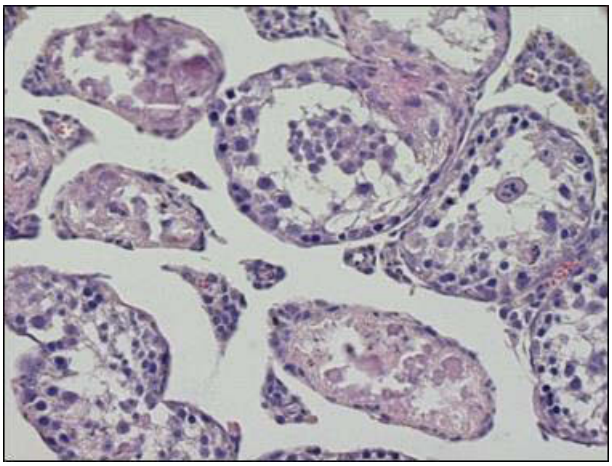

Supplemental 1. Representative micrograph of the testis from Fxr$\alpha^{-/-}$ male exposed to 0.5% CA diet for 1 month.

Discussion et perspectives de l'article I

Dans ce premier article, nous montrons, de façon cohérente avec ce qui est connu dans la littérature (385), que les souris Fxrα$^{-/-}$ présentent une hyper-sensibilité à un régime continu enrichi en acide cholique (perte de poids drastique). Afin de respecter les normes éthiques, nous avons donc adapté le protocole en mettant en place une alternance de régime supplémenté en acides biliaires, et de régime standard. Dans ce contexte, nous montrons que ces animaux montrent une susceptibilité accrue aux effets délétères des acides biliaires, non seulement sur le plan métabolique (poids du corps et des organes du tractus génital), mais également sur la physiologie testiculaire par rapport aux animaux sauvages. L'augmentation de la concentration plasmatique d'acides biliaires, à l'issue d'un mois d'alternance de régime, abouti ainsi à une diminution de la production de spermatozoïdes due à une apoptose des cellules germinales pré- et méiotiques spécifiquement chez les animaux FXRα$^{-/-}$.

1. Par quel(s) mécanisme(s) moléculaire(s) les acides biliaires exercent-ils leurs effets délétères sur la physiologie testiculaire chez les animaux Fxrα$^{-/-}$?

Les travaux précédents de l'équipe (Baptissart *et al*. 2014, Vega *et al.* (*Plos One*) 2015) ont montré que l'augmentation de la concentration plasmatique des acides biliaires, observée en réponse à un régime enrichi en acide cholique chez des animaux C57BL6, s'accompagne d'une augmentation de la concentration intra-testiculaire d'acides biliaires, responsable d'une altération de la physiologie testiculaire par le biais d'une signalisation locale (Tgr5 et Fxrα). *Nous doserons les acides biliaires intra-testiculaires chez les animaux Fxrα$^{-/-}$ nourris en régime contrôle et CA , afin de déterminer si les effets délétères du régime CA observés sur la survie des cellules germinales peuvent être le résultat de l'activation d'une signalisation locale par les acides biliaires*. Dans ce cas, quels acteurs pourraient-ils être impliqués, en l'absence de Fxrα, dans l'apoptose des cellules germinales ?

a) Implication de Tgr5 ?

Nous montrons que la signalisation associée à Tgr5 (Tgr5-Tbx2-Cx43) à l'origine de l'apoptose germinale dans les travaux de Baptissart *et al.* (2014), n'est pas responsable du processus apoptotique observé chez les animaux Fxrα$^{-/-}$ en réponse au régime CA. L'accumulation des messagers des gènes codant Tgr5, Tbx2 et Cx43 n'est en effet pas altérée, tandis que l'intégrité de la barrière hémato-testiculaire est préservée. Cette disparité entre ces deux modèles pourrait s'expliquer par une différence de quantité et / ou de composition du pool d'acides biliaires générées en réponse à l'apport exogène d'acides biliaires. En effet, l'alternance de régime adoptée dans ce travail pourrait conduire à une accumulation moindre d'acides biliaires dans le testicule par rapport à

un régime continu utilisé dans les travaux de Baptissart *et al*. (2014). Par ailleurs, Fxrα contrôle l'expression de nombreux gènes impliqués dans le cycle entéro-hépatique des acides biliaires, notamment ceux codant les enzymes Cyp8b1, et Baat et Bacs, impliquées respectivement, dans la détermination de la composition du pool d'acides biliaires en favorisant la synthèse de l'acide cholique par rapport à l'acide chénodéoxycholique, et dans la conjugaison des acides biliaires. Le pool des acides biliaires et de leur formes conjuguées pourrait ainsi être altéré chez les animaux Fxrα$^{-/-}$ par rapport aux animaux sauvages, et cette altération pourrait perdurer après un régime enrichi en acide cholique. *Le pool d'acides biliaires sera analysé chez les animaux Fxrα$^{-/-}$ exposés au régime enrichi en acides biliaires et comparé à celui publié par Baptissart et al. (2014) afin de clarifier ce point.*

b) Les acides biliaires participent-ils à l'apoptose germinale par l'intermédiaire du stress oxydant chez les animaux Fxrα$^{-/-}$?

Les travaux de Nomoto *et al*. (2009) (386) ont montré que les souris Fxrα$^{-/-}$ sont plus sensibles au stress oxydatif hépatique que les souris sauvages, comme le traduisent l'augmentation de la concentration intra-hépatique des marqueurs de stress oxydatif *8-Hydroxy-2'-Deoxyguanosine* (8-OHdG) et *Thiobarbituric Acid Reactive Substances* (TBARS), ainsi que l'augmentation de l'expression de gènes liés à ce processus (Gsta2, Gstm3, Hmox1, Nqo1 et Mt1). Ils ont par ailleurs établi une corrélation positive entre la concentration hépatique d'acides biliaires et le niveau d'expression des marqueurs du stress oxydatif. Nous pourrions imaginer un phénomène équivalent dans les testicules des animaux Fxrα$^{-/-}$: l'augmentation supposée de la concentration intra-testiculaire d'acides biliaires pourrait ainsi induire des dommages oxydatifs spécifiquement chez les animaux Fxrα$^{-/-}$ participant ainsi à l'apoptose des cellules germinales. *Nous nous proposons donc de doser les espèces réactives oxygénées dans les testicules de souris sauvages et Fxrα$^{-/-}$ en réponse au régime CA, et le cas échéant d'analyser le niveau d'expression de gènes codant des enzymes connues pour être impliquées dans la survenue du stress oxydatif dans cet organe (Glutathione S-Transferase (Gst), Heme Oxygenase (Hmox), NRH : Quinone Oxidoreductase (Nqo)).*

c) Implication des autres récepteurs nucléaires aux acides biliaires : Car, Pxr, Vdr ?

Nous corrélons l'apoptose des cellules germinales, observée en réponse au régime CA spécifiquement chez les animaux Fxrα$^{-/-}$, à un défaut de méiose supposé au vu de la diminution de l'accumulation des messagers des gènes codant Stra8 et Dmc1. L'expression d'autres gènes impliqués dans le déroulement de la méiose, tels que Sycp1 ou Ccna1, n'est en revanche pas altérée. Ces résultats supposent l'intervention de mécanismes d'action plus subtiles et plus ciblés qu'un stress

oxydatif pour induire l'apoptose germinale, et nous ont poussé à envisager l'implication d'autres récepteurs aux acides biliaires (Car, Vdr, Pxr) dans ce processus apoptotique.

La dérégulation de l'expression des gènes codant Stra8 et Dmc1 est associée à l'augmentation de l'expression du répresseur de la différenciation germinale Shp. Ce dernier est une cible connue des récepteurs nucléaires Pxr et Vdr (mais pas de Car), Pxr étant un activateur transcriptionnel de Shp, Vdr un répresseur transcriptionnel (387)-(388). Ces données bibliographiques nous ont poussées, parmi ces trois récepteurs, à nous focaliser sur Pxr.

2. Pxr, le médiateur des acides biliaires chez les animaux Fxrα$^{-/-}$?

Nous montrons que l'activation *in vivo* de Pxr par un agoniste synthétique, le *Pregnenolone-16α-Carbonitrile* (PCN), semble reproduire la dérégulation de la voie de signalisation Shp-Stra8/Dmc1, suggérant l'implication potentielle de ce récepteur dans l'effet pro-apoptotique des acides biliaires. Cependant, le traitement PCN n'induit pas, après 4 jours, d'augmentation de l'apoptose germinale.

a) Pxr induit-il l'apoptose germinale sur le long terme ?

Quatre jours de traitement PCN sont peut être trop courts pour déclencher un processus apoptotique. *Nous proposons d'initier un traitement PCN in vivo sur une durée plus longue, équivalente à celle utilisée pour le régime CA, soit un mois. Nous procèderons alors à une analyse TUNEL afin de mettre en évidence ou non la présence de cellules germinales en apoptose.*

b) Quel ligand pour Pxr ?

Parmi les acides biliaires, le ligand préférentiel de Pxr est le LCA. Celui-ci est présent dans le plasma, mais pas détecté dans le testicule de souris C57BL6 nourries en régime contrôle ou en régime enrichi en acide cholique (Baptissart *et al*. 2014, Vega *et al.* (*Plos One*) 2015). Dans ce contexte, par quel ligand Pxr serait-il activé dans le testicule ? L'analyse des souris dont le gène codant l'enzyme impliquée dans la synthèse des acides biliaires Cyp27a1 a été invalidé apporte un élément de réponse à ce questionnement (389)-(390). En effet, ces animaux présentent une accumulation de précurseurs des acides biliaires dans l'organisme, responsables de l'activation de la signalisation associée à Pxr comme le suggère l'augmentation de l'accumulation hépatique des transcrits de gènes cibles connus de ce récepteur (Cyp3a11, Mrp2, Oatp2). De telles molécules intermédiaires sont-elles synthétisées dans le testicule ? Cette hypothèse est soutenue par les travaux de Smith *et al*. (2009) menés chez l'Homme (391). Les auteurs ont mis en évidence la présence d'acides biliaires dans le fluide follicullaire à une concentration supérieure à celle retrouvée dans le plasma, ainsi que des enzymes nécessaires à leur synthèse dans les cellules de Granulosa (CYP7A1 pour la voie classique,

CYP27A1 et CYP7B1 pour la voie alternative). Ces enzymes sont par ailleurs fonctionnelles : des cellules de granulosa humaines en culture sont en effet capables de synthétiser des acides biliaires en réponse à un apport de cholestérol. Les cellules de Leydig étant les ''homologues'' des cellules de Granulosa, elles pourraient être la source d'une synthèse locale d'acides biliaires. Ce sont en effet des cellules stéroïdogènes dotées d'une réserve importante de cholestérol, le substrat de la synthèse des acides biliaires. Elles expriment par ailleurs, comme les cellules de Granulosa humaines, les récepteurs nucléaires connus pour contrôler la synthèse des acides biliaires : Fxrα, Shp, Lxr, Lrh-1. Afin de tester cette hypothèse, nous nous proposons de travailler avec une culture primaire de cellules de Leydig murine:

1- nous vérifierons la présence des transcrits des gènes codant les enzymes impliquées dans les différentes étapes de la voie de biosynthèse des acides biliaires dans ces cellules (Cyp7a1, Cyp27a1, Cyp7b1, 3ϐSd7, Cyp8b1, Akr1d1) ;

2- nous doserons les acides biliaires potentiellement synthétisés dans les cellules et sécrétés dans le milieu de culture en réponse à l'apport de cholestérol. Si tel est le cas, nous nous proposons par ailleurs de comparer le niveau d'expression des enzymes détectées, et la quantité d'acides biliaires synthétisées dans les cellules de Leydig isolées de souris sauvages et de souris Fxrα$^{-/-}$ afin de déterminer si comme dans le foie, la synthèse testiculaire des acides biliaires est dépendante de Fxrα.

Toutefois, dans l'hypothèse où Pxr serait activable dans le testicule des souris Fxrα$^{-/-}$ en réponse au régime CA par des précurseurs des acides biliaires, cela sous-entendrait que la synthèse d'acides biliaires soit interrompue permettant une accumulation de ces précurseurs. Deux possibilités s'offrent alors : soit l'ensemble des enzymes impliquées dans la synthèse des acides biliaires ne sont pas exprimées dans les cellules du testicule, soit l'expression de ces enzymes est dérégulée dans le testicule comme dans le foie des souris Fxrα$^{-/-}$.

3. Pxr et testicule

Le récepteur nucléaire Pxr est majoritairement connu pour son rôle dans l'élimination de l'organisme de substances toxiques qui lui servent de ligand : les xénobiotiques et les endobiotiques. Notre étude est l'une des rares a avoir identifié la présence de Pxr dans le testicule, et a avoir montré que sa signalisation est active dans cet organe. Elle ouvre donc des perspectives intéressantes quant-au rôle potentiel de Pxr dans le testicule.

a) Où est Pxr dans le testicule ?

L'analyse du niveau d'expression de Pxr dans des échantillons de testicule total et de tri cellulaire de cellules germinales montre qu'il est enrichi dans certains stades germinaux (spermatocytes pachytènes et spermatides rondes) par rapport au testicule total. Ces données n'excluent cependant pas qu'il puisse être également exprimé dans les types cellulaires somatiques du testicule (Leydig, Sertoli, Péritubulaires). Pxr pourrait alors être impliqué dans le contrôle d'autres processus testiculaires que celui de la différenciation germinale décrit dans ce travail. *Une analyse plus précise de l'expression de Pxr dans le testicule (ontogénie, immunohistochimie) devrait être conduite afin de déterminer avec précision le(s) type(s) cellulaire(s) dans le(s)quel(s) Pxr est exprimé.*

b) Pxr, un nouvel acteur de la physiologie testiculaire ?

Les souris dont le gène codant Pxr a été invalidé se reproduisent normalement (392), suggérant que la perte de ce récepteur n'est pas essentielle à la physiologie testiculaire. Son activation semble elle être délétère. En effet, nous montrons que l'utilisation *in vivo* de son agoniste synthétique PCN induit après 4 jours de traitement une augmentation de Shp associée à une diminution de l'accumulation des transcrits de Stra8 et Dmc1, et pourrait ainsi altérer la différenciation germinale. Dans ce contexte d'activation, Pxr joue-t-il un rôle dans d'autres processus testiculaires ? Pour répondre à cette question, *il serait nécessaire d'étudier l'impact du PCN sur d'autres aspects de la physiologie testiculaire (activité endocrine des cellules de Leydig, fonctions Sertoliennes ...), ainsi que de réaliser de nouvelles cinétiques d'exposition in vivo au PCN afin notamment d'étudier l'impact de l'activation de Pxr sur le long terme sur la fertilité des animaux.* Par ailleurs, une fois la localisation de Pxr précisée dans le testicule, *la sur-expression de ce récepteur spécifiquement dans le(s) type(s) cellulaire(s) concerné(s) permettrait de s'affranchir d'éventuels effets métaboliques du PCN pouvant altérer la physiologie testiculaire.*

c) Pxr et détoxication dans le testicule.

Le récepteur nucléaire Pxr a un rôle central dans le métabolisme des xénobiotiques et des endobiotiques. Il active en effet l'expression d'un panel de gènes codant des enzymes de détoxication (parmi lesquelles des membres des familles Cyp1a, Cyp2b, Cyp2c, Cyp3a, gluttathione-S-transferase, UDP-glucuronosyltransferase, sulfotransferase...) ainsi que des transporteurs (*Multidrug Resistance* (Mdr), *Multidrug Resistance-associated Protein* (Mrp), Oatp) qui permettront l'élimination des substances toxiques de l'organisme. Certains de ces gènes sont exprimés dans le testicule (dans les cellules de Leydig, de Sertoli (BHT) et les spermatides rondes), et pourraient ainsi limiter la toxicité de ces molécules sur les activités endocrine et exocrine testiculaires sous l'influence de Pxr. Ainsi Pxr pourrait-il participer à l'élimination des xénobiotiques du testicule ? Comme précisé ci-dessus, la perte de Pxr ne semble pas entraîner de phénotype d'infertilité dans un contexte

physiologique. Qu'en serait-il à la suite d'une exposition à un xénobiotique / endobiotique. On observe depuis plusieurs années une augmentation significative de pathologies testiculaires (infertilité, cancers). L'exposition environnementale à de nombreuses substances (pesticides...) est suspectée de participer à l'étiologie de telles pathologies. Une dérégulation de l'expression et / ou de l'activité de Pxr pourrait-elle participer, en combinaison avec de telles expositions et *via* un défaut d'élimination de ces substances toxiques, à l'étiologie de ces pathologies ? *L'analyse de souris, dont le gène codant Pxr a été invalidé spécifiquement dans le testicule, exposées à de telles molécules serait très intéressante en ce sens. Si tel est le cas, la recherche de polymorphismes dans la région génomique du gène codant PXR chez des patients souffrant de telles pathologies testiculaires pourrait être envisagée* : PXR pourrait alors être identifié comme un nouvel acteur à l'origine de pathologies testiculaires en relation avec un facteur environnemental, et désigné comme une nouvelle cible thérapeutique pour le traitement de ces pathologies.

3. Shp est-il bien impliqué dans les effets de Pxr ?

Nous montrons que l'activation de Pxr entraîne une diminution de l'expression de Stra8 et Dmc1, associée à une augmentation de l'expression de Shp. Ce dernier est-il bien l'intermédiaire entre Pxr et l'altération méiotique observée ? *Nous nous proposons d'exposer des souris sauvages et Shp$^{-/-}$ au PCN et d'analyser l'expression de Stra8 et Dmc1 en réponse à cette exposition.*

4. Polymorphismes de FXRα et pathologies hépatiques : un contexte favorable à l'infertilité masculine ?

Des données cliniques ont permis d'établir un lien entre des pathologies hépatiques, et des troubles de la fertilité masculine (393)-(394)-(395). Ces données sont par ailleurs soutenues par l'analyse de modèles animaux de cirrhose induite ou encore de ligature des voies biliaires, révélant des altérations histologiques testiculaires consistant respectivement en la destructuration des tubes séminifères et la perte de la lignée germinale (396)-(397). Les travaux de Baptissart *et al*. (2014) ont montré que l'augmentation de la concentration plasmatique d'acides biliaires, qui est le plus précoce et le plus sensible des paramètres biochimiques observés en cas de pathologies hépatiques (398), est responsable de l'apparition d'un phénotype d'infertilité. Nous montrons dans ce premier article que les animaux Fxrα$^{-/-}$ sont plus sensibles aux effets délétères des acides biliaires sur la physiologie testiculaire que les souris sauvages. Ce travail définit donc la perte de fonction du récepteur nucléaire Fxrα comme un contexte plus favorable au développement de troubles de la fertilité dans le cas de pathologies hépatiques. *La recherche de polymorphismes dans la région génomique du gène codant FXRα associée à une perte d'expression ou de fonction de ce récepteur chez des patients*

atteints d'hépatopathies pourrait ainsi servir de marqueur pronostic et / ou diagnostic pour la survenue d'une stérilité.

Article II

Bile acid-FXRα pathways regulate male sexual maturation in mice.

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La puberté est une étape clé quant-à la mise en place des fonctions reproductives mâles. Elle correspond à la mise en route de l'axe hypothalamo-hypophyso-gonadique, permettant la synthèse d'androgènes impliqués dans le développement complet des caractères sexuels et l'initiation de la spermatogenèse. Cette phase développementale est donc particulièrement sensible aux perturbations endocriniennes.

Fxrα est un récepteur nucléaire qui a récemment été identifié comme un nouvel acteur impliqué dans le contrôle de l'activité endocrine des cellules de Leydig, et plus généralement dans le contrôle du métabolisme des androgènes. Il est exprimé dans le testicule, majoritairement dans les cellules de Leydig, dans lesquelles son activation *in vivo* par un agoniste synthétique conduit, après 12 heures de traitement, à la répression de la stéroïdogenèse (33). De plus, les données présentées dans l'article I montrent que Fxrα participe au catabolisme hépatique de la testostérone. Ainsi, la dérégulation de la signalisation associée à Fxrα au cours de la puberté pourrait-elle perturber la maturation sexuelle ?

Bile acid-FXRα pathways regulate male sexual maturation in mice

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ABSTRACT

The bile acid receptor Farnesol-X-Receptor alpha (FRXα) is a member of the nuclear receptor superfamily. FRXα is expressed in the interstitial compartment of the adult testes, which contain the Leydig cells. In adult, short term treatment (12 hours) with FRXα agonist inhibits the expression of steroidogenic genes *via* the induction of the Small heterodimer partner (SHP). However the consequences of FRXα activation on testicular pathophysiology have never been evaluated. We demonstrate here that mice fed a diet supplemented with bile acid during pubertal age show increased incidence of infertility. This is associated with altered differentiation and increase apoptosis of germ cells due to lower testosterone levels. At the molecular level, next to the repression of basal steroidogenesis via the induction expression of *Shp* and *Dax-1*, two repressors of steroidogenesis, the main action of the BA-FRXα signaling is through lowering the Leydig cell sensitivity to the hypothalamo-pituitary axis, the main regulator of testicular endocrine function. In conclusion, BA-FRXα signaling is a critical actor during sexual maturation.

INTRODUCTION

The nuclear receptor Small Heterodimer Partner (SHP) have been demonstrated to control sexual maturation in male mice [1]. SHP is a known target gene of the nuclear bile acid receptor Farnesol-X-Receptor-α (FRXα). However, the potential roles of BA on male sexual maturation have never been studied so far. Interestingly, experimental models of liver injury show altered puberty with primary hypogonadism [2]. It is known that such conditions of liver disorders lead to increased bile acid levels. We hypothesized that BAs could alter male sexual maturation during puberty *via* FRXα.

Puberty is a key event for the establishment of male reproductive functions. Puberty depends on the increase of testosterone levels which is under the control of the hypothalamo-pituitary axis activity. This leads to the maturation of secondary sexual characteristics, the establishment and the maintenance of spermatogenesis and then fertility [3].

In the present study, we first analyzed the impact of pubertal BA-exposure on testicular physiology. In order to decipher the involved molecular mechanisms, we used a classical approach with diet supplemented with BA (cholic acid 0.5%) [4]. We demonstrate that pubertal mice fed a diet supplemented with cholic acid (CA) have altered fertility associated with default in germ cell differentiation correlated with an increased rate of spermatocytes apoptosis. We have validated that these effects are not mediated by TGR5. This is due to altered testosterone synthesis. Surprisingly these impacts of BA were not fully mediated by SHP. Interestingly,

we pinpoint that the gene encoding the nuclear receptor *Dax-1* (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene-1), a repressor of steroidogenesis and a related to SHP, is a target of FRXα. Moreover, we defined a major impact of BA-exposure explaining the impact on testicular endocrine function. Indeed, in vivo and in vitro approaches demonstrated that FRXα activation decreases Leydig cells sensitivity to the hypothalamo-pituitary axis signaling. Using pharmacological experiments we have established that the effect of BAs is mainly due to the transcriptional repression of the gene encoding the luteinizing hormone receptor (Lhcgr). BA levels are increased during liver diseases, thus these results, in combination with previous study in adult, highlight the complexity of the interaction between the liver and testicular functions throughout lifetime.

RESULTS

Dietary BA supplementation alters male fertility

To identify links between pubertal BA-exposure and male fertility, mice were fed a normal control diet supplemented with 0.5% of cholic acid (CA). CA-diet led to altered fertility with 60% of the exposed males unable to give progeny (Figure 1A). In males giving progenies, CA-diet also decreased the number of pups per litter (Figure 1B). The combination of the increase of sterile males and decrease of pups per litter resulted in a 60% decrease of the number of pups generated by CA-exposed males compare to control-diet group (Figure 1C). This decreased fertility was associated with a lower production of spermatozoa as revealed by the counting of sperm number in the epididymis head and tail (Figure 1D).

BA-diet alters postnatal growth

The impact of BA-diet on fertility is associated with altered postnatal growth as mice exposed to CA-diet present lower weight gain starting 5 days after the beginning of the treatment (Figure 2A). CA-exposed males showed reduced body length (Figure 2B). However, the overall food intake was not altered (Figure 2C). The altered postnatal growth during this pubertal period affects male genital tract with lower weight gain in testis, epididymis and seminals (Figure 2D), whereas the liver weight was not affected (Figure 2D). In addition,it must be noticed that the liver weight relative to body weight was increased suggesting liver injury (Supplementary Figure S1A); which is consistent with alteration of genes such as Cyp3a25 and Sult2a1 (Supplementary Figure S1B) and the increase of plasma BA levels after 5 days of CA-diet exposure (Figure 2E). In Figure 2D, weights are represented with chow-diet group as reference (100%) at particular age. It has to be noticed that organ gross weights increased during postnatal development (between 5 days post-treatment (dpt) and 44dpt), the increase is less pronounced in CA-diet treated mice (Supplementary Figure S1C) which sustained the impact of CA-diet on global postnatal growth. In addition, weights relative to body weight of epididymis and seminals was also affected (Supplementary Figure S2D).

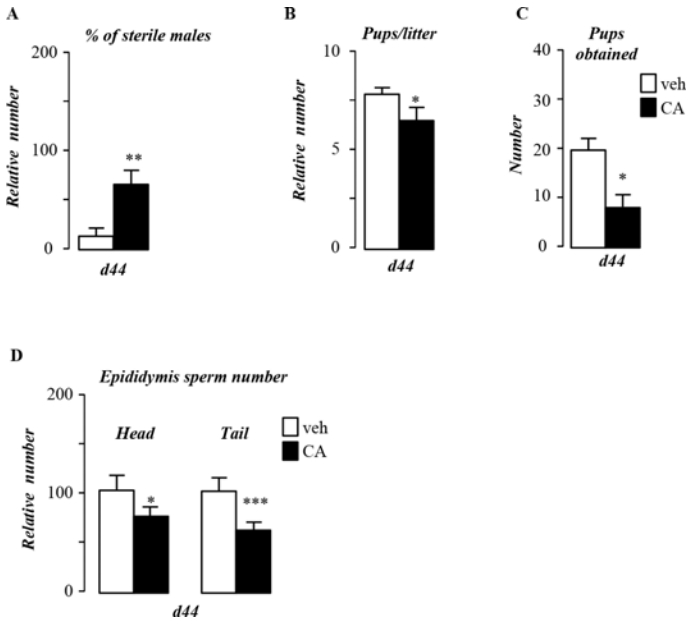

Figure 1: Pubertal exposure to BA alters male fertility. A. Percentage of infertile males. **B.** Number of pups per litter. **C.** Total number of pups obtained per group. **D.** Sperm count in the epididymis head and tail of control or CA fed groups for 44 days. In all of the panels data are expressed as the means ± SEM. Statistical analysis:*, $p<0.05$; **, $p<0.01$, ***, $p<0.005$ *vs.* control diet group.

Pubertal BA-exposure alters germ cell survival

Histological analyses of testis showed that BA-exposed mice showed altered germ cell differentiation as visualized with a decrease in the number of seminiferous tubules with elongated spermatid cells (Figure 3A & 3B). Analysis of the expression of pre-meiotic (*Plzf*, *G9a, Stra8*,), meiotic (*Dmc1*, *Mei1)* and post-meiotic (Prm2) genes showed peculiar kinetic of events. Consistent with the observed decrease of post-meiotic cells at 44dpt (day post beginning of the treatment) the expression of *Prm2* was lower in CA-treated group compare to control group (Figure 3C). Interestingly at this age the pre-meiotic and meiotic genes were not affected at that age (Supplementary Figure S2A). This delay in germ cell differentiation was correlated with an early apoptotic wave of germ cells after 5-days of CA-diet (Figure 3D & 3E). This apoptotic wave was transient as at 44-days after the beginning of the treatment no more difference was observed between groups (Figure 3E). In contrast, 5 days after the beginning of the treatment, neither the mRNA accumulation of pre-meiotic and meiotic genes such as *Plzf*, *G9a*, *Stra8*, *Dmc1* and *Mei1* nor the post meiotic gene, Prm2 were significantly affected by the CA-diet (Figure 3F). Thus the altered meiosis process was not due to the altered expression of key meiotic genes such as *Stra-8* and *Dmc1*.

BA-diet induces germ cell death via alteration of testosterone metabolism

Germ cell death has previously been associated with androgen withdrawal [5]. Interestingly, CA-exposed males, for 5 days, showed a decrease of intra-testicular levels of testosterone (Figure 4A). This was associated with a decrease of the *Steroidogenic acute regulatory protein* (*Star)* mRNA accumulation in testis of CA-exposed mice compare to control group (Figure 4B). No statistically significant effect was found on testosterone levels after 44 days of CA-diet (Supplementary Figure S2B), whereas star mRNA accumulation was still decrease in CA-exposed males (Supplementary Figure S2C). The involvement of testosterone decrease in germ cell death was sustained by the fact that supplementation with testosterone counteracted the effect of CA-diet on germ cell apoptosis (Figure 4C). In order to decipher if testosterone impacted *per se* germ cell physiology or if it could act *via* its aromatization into estradiol, we studied the estrogenic signaling pathways, which are known regulators of steroidogenesis in Leydig cells [6], [7]. Intra-testicular levels of estradiol were not affected by CA-diet after 5-days of exposure (Figure 4D), a time when germ cell apoptosis was seen. We next analyzed

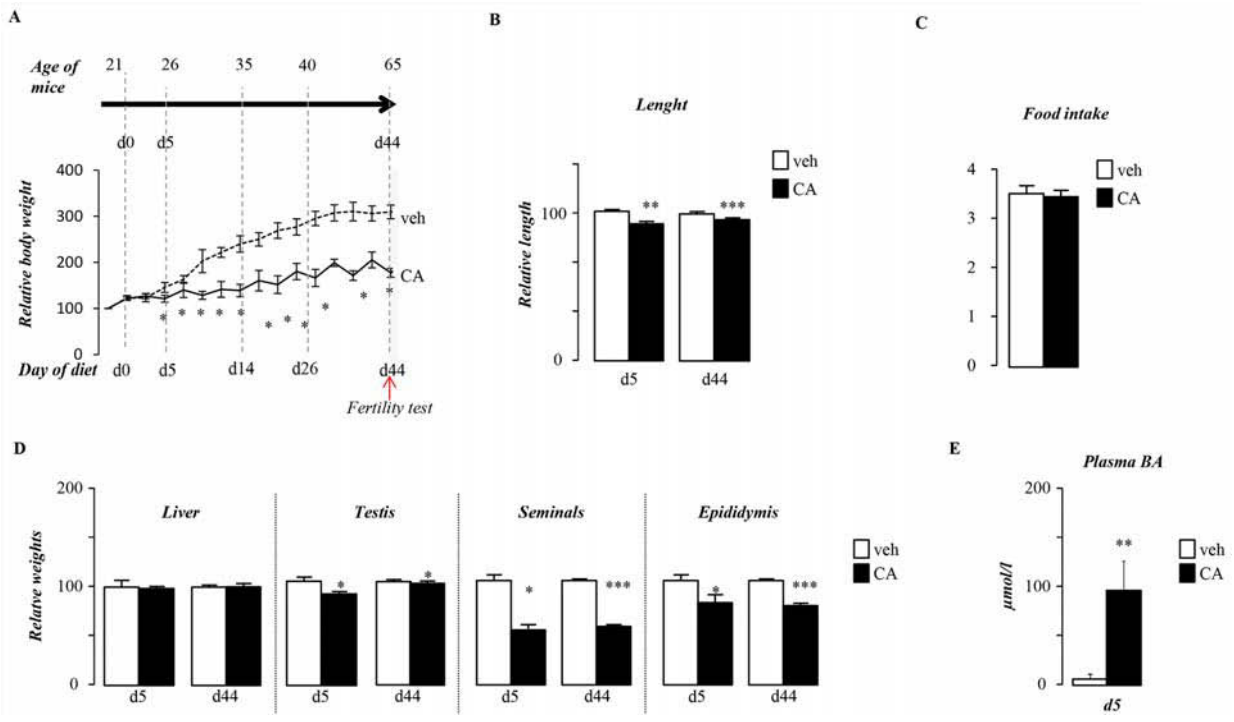

Figure 2: Pubertal exposure to BA alters pubertal growth. A. Overall body weight gain of males exposed to control or CA diets. **B.** Body length of males after 5 or 44 days of either control or CA diet. **C.** Overall food intake of male fed control or CA diet. **D.** Liver, testis, epididymis and seminal relative gross weights in C57Bl/6J mice fed 5 or 44 days of exposure. **E.** Plasma bile acid levels in males exposed to control or CA diets for 5days. In all of the panels data are expressed as the means ± SEM. Statistical analysis: *, $p<0.05$; **, $p<0.01$, ***, $p<0.005$ *vs.*respective control group.

the expression of the two estrogen receptors described in Leydig cells, the G protein coupled receptor *Gpr30* [7] and the nuclear receptor estrogen receptor alpha (*Erα*) [8]. If the expression of *Gpr30* was not altered, the CA-exposed males showed a lower level of *Erα* compared to control group (Figure 4E). This impact on *Erα* expression was supported by the altered expression of testicular ERα target genes such as Renin-1 (Figure 4F). These results suggest that estrogenic pathway must be altered in the context of CA-diet. In order to discriminate its involvement in CA-induced germ cell apoptosis, we used specific antagonists of GPR30 or ERα, respectively G15 [9] and ICI 182, 780 [10]. None of these antagonists was able to counteract the effect of CA on germ cell death (Figure 4G).

BA-diet alters germ cell survival in TGR5 independent pathways

Consistent with what was previously demonstrated, exposure to BA-diet led to increase of intra-testicular BA levels (Figure 5A). In contrast to the adult BA exposure [11], our data clearly demonstrate that TGR5 was not involved in the pubertal phenotype induced by BA-exposure as *Tgr5*$^{-/-}$ mice showed altered fertility associated with higher apoptotic germ cell (Supplementary Figure S3A, S3B, S3C & S3D) in response to CA-diet. The lack of role of TGR5 is sustained by the fact that the mRNA accumulation of Connexin-43, a testicular TGR5 target gene [11], was not altered in the present model (Supplementary Figure S3E).

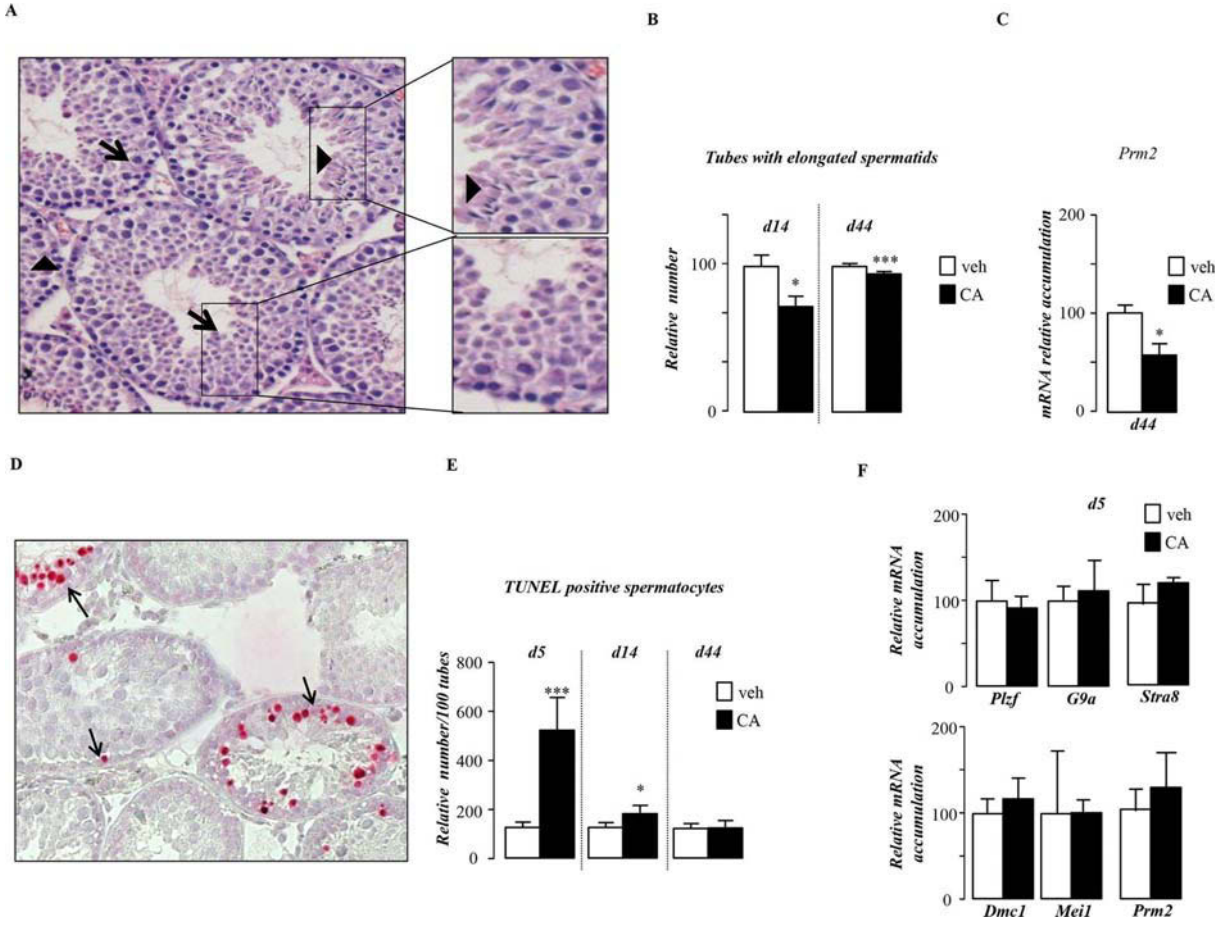

Figure 3: Pubertal exposure to BA alters germ cell survival. A. Representative micrographs of hematoxylin/eosin-stained testes of mice fed CA-diet for 14 days. The arrow-head indicates tubules with elongated spermatids; arrows indicate tubes without elongated spermatids. The original magnification was x200. **B.** Quantification of the number of tubules with elongated spermatids per 100 seminiferous tubules after 14 and 44 days of control or CA-diet (n=10-20 per group). **C.** Testicular mRNA expression of *Prm2* normalized to *β-actin* levels in whole testis of C57Bl/6J mice fed control or CA diets for 44 days (n=10 to 15 per group). **D.** Apoptosis in mice exposed to control or CA diets (n=10-20 per group) analyzed by TUNEL staining. Representative micrographs of testis exposed to control or CA diets for 5 days. The arrows indicate apoptotic spermatocytes. The original magnification was x200. **E.** Quantification of TUNEL analyses after 5, 14 or 44 days of diet exposure. The number of TUNEL-positive is indicated as the number of positive cells per 100 seminiferous tubules (n=10-20). **F.** Testicular mRNA expression of *Plzf, G9a, Stra8, Dmc1, Mei1* and *Prm2* normalized to *β-actin* levels in whole testis of C57Bl/6J mice fed control or CA diets for 5 days (n=10 to 15 per group). Control diet treated mice were arbitrarily fixed at 100%. In all of the panels data are expressed as the means ± SEM. Statistical analysis: *, p<0.05; **, p<0.01, ***, p<0.005 *vs.*respective control group.

BA-diet alters pubertal testicular physiology through FRXα

Due to the hyper-sensibility of Fxrα-/- mice to CA-diet, these mice could not be exposed to CA-diet as high mortality levels was observed even after 5-days of exposure. Thus to decipher the potential involvement of FXRα in the impact of BA-diet, males were thus exposed to FXRα synthetic agonist, GW4064. 5-days of exposure with GW4064 days repressed testosterone synthesis (Figure 5B) associated with lower *Star* mRNA accumulation (Figure 5C). This in turn led to an increase of germ cell apoptosis (Figure 5D). This supports the idea that FXRα was involved in the observed phenotype. Consistently, no decrease of testosterone levels was observed in mice invalidated for the gene encoding FXRα (Fxrα-/-) in response to GW4064 (Figure 5E). In that line no effect of GW4064 was observed on *Star* mRNA accumulation in Fxrα-/- males (Figure 5F). Moreover, no impact of GW4064 on the number of apoptotic cells was observed in Fxrα-/- mice (Figure 5G).

FXRα-BA-diet alters germ cell survival in SHP independent pathways

We next wanted to analyze the involvement of the small heterodimer partner receptor (SHP), a known target gene of FXRα, which has been demonstrated to repress steroidogenesis [1]. In the present study, *Shp* expression was increased by CA-diet during

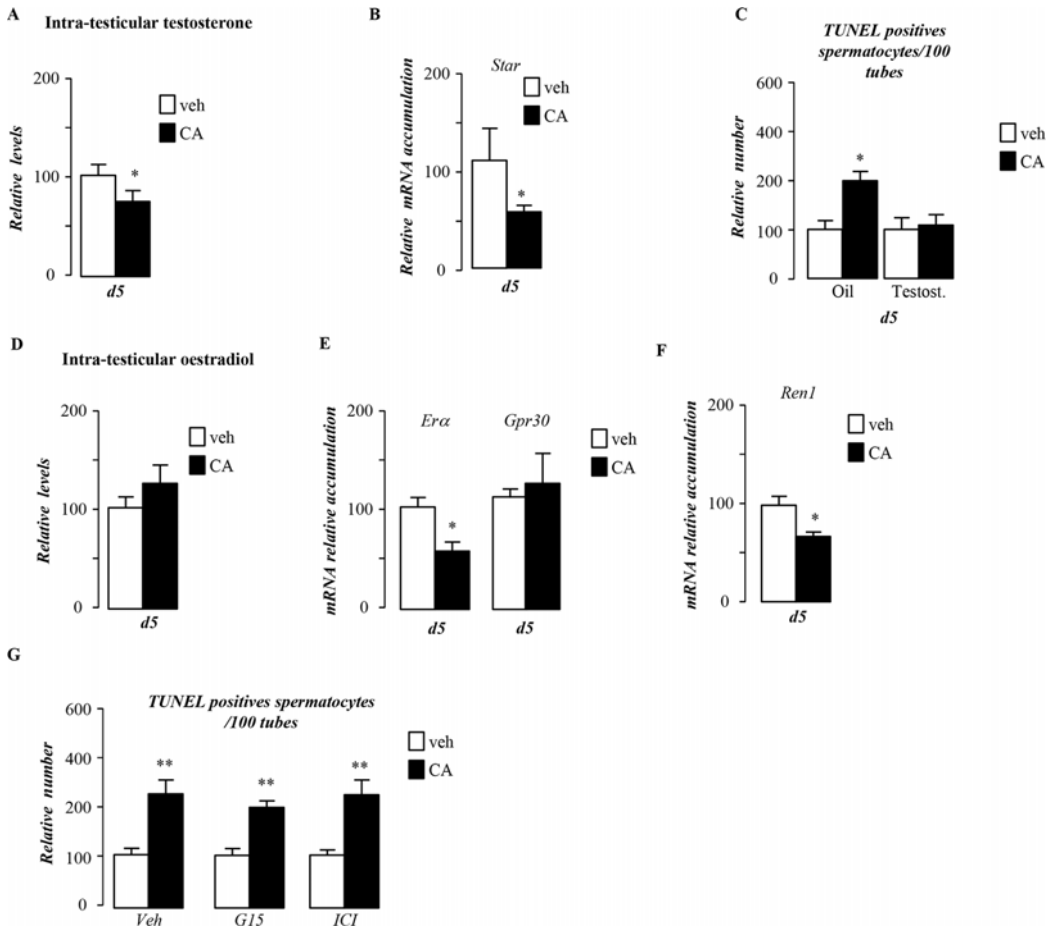

Figure 4: Pubertal BA exposure regulates testicular androgen metabolism. A. Relative intra-testicular testosterone levels in C57Bl/6J mice fed control or CA diet for 5 days (n=6-20 per group). **B.** Testicular mRNA expression of *Star* normalized to *β-actin* levels in whole testis of C57Bl/6J mice fed control or CA diets for 5 days (n=10 to 15 per group). **C.** Quantification of TUNEL analyses after testosterone or vehicle treatment in males fed control or CA diets for 5 days. The number of TUNEL-positive spermatocytes is indicated as the number of positive cells per 100 seminiferous tubules (n=10-20). **D.** Relative intra-testicular estrogen levels in C57Bl/6J mice fed control or CA diet for 5 days (n=6-20 per group). **E.** Testicular mRNA expression of *Erα* and *Gpr30* normalized to *β-actin* levels in whole testis of C57Bl/6J mice fed control or CA diets for 5 days (n=10 to 15 per group). **F.** Testicular mRNA expression of *Renin-1* normalized to *β-actin* levels in whole testis of C57Bl/6J mice fed control or CA diets for 5 days (n=10 to 15 per group). **G.** Quantification of the number of TUNEL positive cells per 100 seminiferous tubules after 5 days of control or CA-diet co-treated with either vehicle, G15 or ICI (n=5-10 per group). In all panels control diet or vehicle treated group were arbitrarily fixed at 100% and data are expressed as the means ± SEM. Statistical analysis: *, $p<0.05$; **, $p<0.01$, ***, $p<0.005$ *vs.* respective control group.

pubertal period (Figure 6A). Consistent with previous work the increase of *Shp* was associated with a lower accumulation of *Lrh-1* mRNA, a known inducer of steroidogenesis (Figure 6A). In contrast, the expression of *Sf1* was not affected (Figure 6A). We studied the role of SHP in the testicular phenotype during juvenile cholestasis using knock-out mice. Surprisingly, $Shp^{-/-}$ males exposed to CA-diet showed altered fertility (Figure 6B & 6C). In addition, as in wild-type, CA-diet led to a lower testosterone level (Figure 6D) associated with an increased germ cell apoptosis (Figure 6E) in $Shp^{-/-}$ males. This suggests that during puberty, BAs can repress testicular steroidogenesis in a SHP-independent manner.

Dax-1 is a direct target gene of FXRα/RXR heterodimer

As *Shp* deficiency was not sufficient to reverse the impact of BA exposure on testosterone synthesis, we wondered whether other repressor of steroidogenesis could be involved. We focused on DAX-1 which is closely related to SHP. In order to validate if *Dax-1* is a *bona fide* target gene of FXRα, we used GW4064. Treatment with GW4064 resulted in an increased of testicular mRNA accumulation of both *Shp* and *Dax-1* (Figure 7A). As Dax-1 is expressed in several cell types of the testis [12, 13], we wanted to ensure that the effect of GW4064 was on Leydig cells. *In vivo* we demonstrate that FXRα is mainly expressed in interstitial compartment of the testis as ensure by the analysis of the expression of specific markers such as *Lhcgr* (Leydig) and *Fshr* (Sertoli) or *Oct3/4* (germ cells) (Figure 7B). As for *Lhcgr*, the expression of *Fxrα* was enriched in interstitial samples. In contrast, *Fshr* and *Oct3/4* only show slight increase in tubular and not in Intestitial samples. The slight increase of *Fshr* and *Oct3/4* might be explained by the fact that Sertoli and Oct3/4 positive spermatogonia represent a small percentage of cells within the seminiferous tubules as these samples contained peritubular cells as well as differentiating spermatogonia, primary and secondary spermatocytes as well as post-meiotic germ cells. MA10 Leydig cells treated

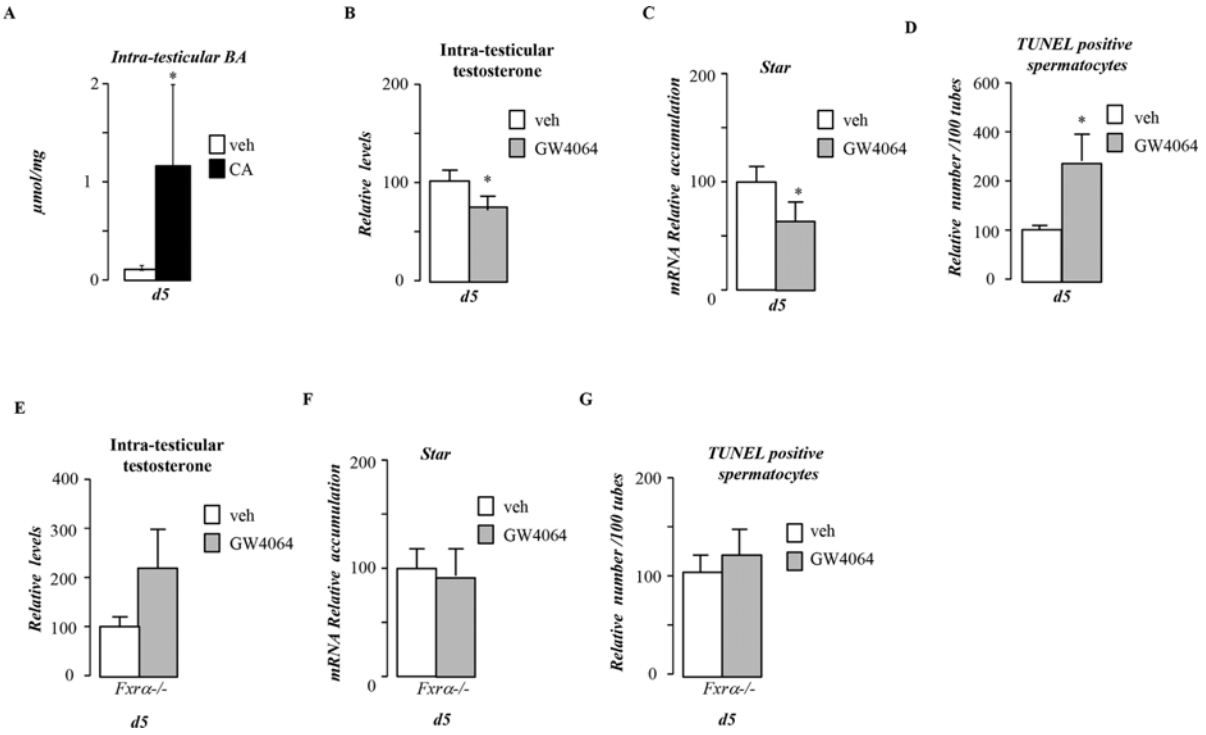

Figure 5: Pubertal BA exposure impacts testis physiology via FXRα. A. Relative intra-testicular bile acid levels in *C57Bl6*.mice fed control or CA diet for 5 days (n=5-6 per group). **B.** Relative intra-testicular testosterone levels in *C57Bl6*.mice treated with vehicle or GW4064 for 5 days (n=5-6 per group). **C.** Testicular mRNA expression of *Star* normalized to *β-actin* levels in whole testis of C57Bl/6J mice treated with vehicle or GW4064 for 5 days (n=10 to 15 per group). **D.** Quantification of TUNEL analyses in males treated with vehicle or GW4064 for 5 days. The number of TUNEL-positive spermatocytes is indicated as the number of positive cells per 100 seminiferous tubules (n=10-20). **E.** Relative intra-testicular testosterone levels in *Fxrα-/- male* mice treated with vehicle or GW4064 for 5 days (n=5-6 per group). **F.** Testicular mRNA expression of *Star* normalized to *β-actin* levels in whole testis of *Fxrα-/- male* mice mice treated with vehicle or GW4064 for 5 days (n=10 to 15 per group). **G.** Quantification of TUNEL analyses in *Fxrα-/- male* mice treated with vehicle or GW4064 for 5 days. The number of TUNEL-positive spermatocytes is indicated as the number of positive cells per 100 seminiferous tubules (n=10-20). In all panels control diet or vehicle treated group were arbitrarily fixed at 100% and data are expressed as the means ± SEM. Statistical analysis: *, $p<0.05$; **, $p<0.01$, ***, $p<0.005$ *vs.*respective control group.

with GW4064 showed similar increased of *Dax-1* mRNA accumulation after 12 hours of treatment (Figure 7C). *In silico* analysis of 5'-sequences of the *Dax-1* mouse and human genes revealed a putative FXRE sequence (IR1) (Figure 7D). Ability of FXRα to transactivate the promoter of *hDAX-1* was assessed. Ectopic expression of RXR/FXRα by transient transfection elicited 1.2kb-h*DAX-1* promoter activity in a ligand-dependent manner as shown by the use of GW4064 (Figure 7E). Direct interaction of the RXR/FXRα heterodimer with the FXRE was confirmed by electromobility shift assays. A significant band shift was observed when both FXRα and RXR were added, which was specifically competed away by a 200-fold molar excess of the unlabeled consensus IR1 sequence of FGF19gene ; whereas nonspecific sequence (LXRE [14]) did not compete the binding of the heterodimer. (Supplementary Figure S4A).

Using Fxrα-/- males exposed 5 days to GW4064, *Shp* and *Dax-1* were confirmed as FXRα target within the testis (Figure 7F).

In order to define if DAX-1 is involved in the effect of FXRα on testicular steroidogenesis, we analyzed the impact of GW4064 in MA-10 cells transfected with a siRNA directed against Dax-1. Data show that DAX-1 is involved in the impact of GW4064 on steroidogenesis as supported by the lack of impact of GW4064 on steroid synthesis and *Star* mRNA accumulation in cell treated with a specific siRNA directed against *Dax-1*(Figure 7G & 7H). Combined these data suggest that DAX-1 is involved for the effect of the FXRα-GW4064 impact on basal testicular steroidogenesis.

BA exposure reduces Leydig cell sensitivity to LH/CG

In addition, we demonstrate that *in vivo Lhcgr* mRNA accumulation was altered by FXRα-signaling pathways using either CA-diet or GW4064 exposures (Figure 8A). This effect was mediated by FXRα as no effect of GW4064 on Lhcgr was observed in Fxrα-/- males (Figure 8B). This effect was also observed in MA10 cells (Figure 8C). These data suggest that exposure to FXRα agonist could alter the sensitivity of Leydig cells to luteinizing hormone/chorionic gonadotropin (LH/CG). We tested this hypothesis *in vivo* and *in vitro*. For that purpose, male mice were exposed to GW4064 for 5 days and were injected with 5IU of hCG for 12 hours. In vehicle treated mice, hCG induced a 10-fold increase of testosterone levels within testis (Figure 8D) associated with a 2-fold increase at plasma levels (Figure 8E). In contrast, the GW4064 pre-exposed males showed only a 5 fold increase of testicular levels of testosterone and no elevation in the plasma (Figure 8D & 8E). This was sustained by the lower effect of hCG on *Star* mRNA accumulation in GW4064 treated group compare to vehicle one (Figure 8F).

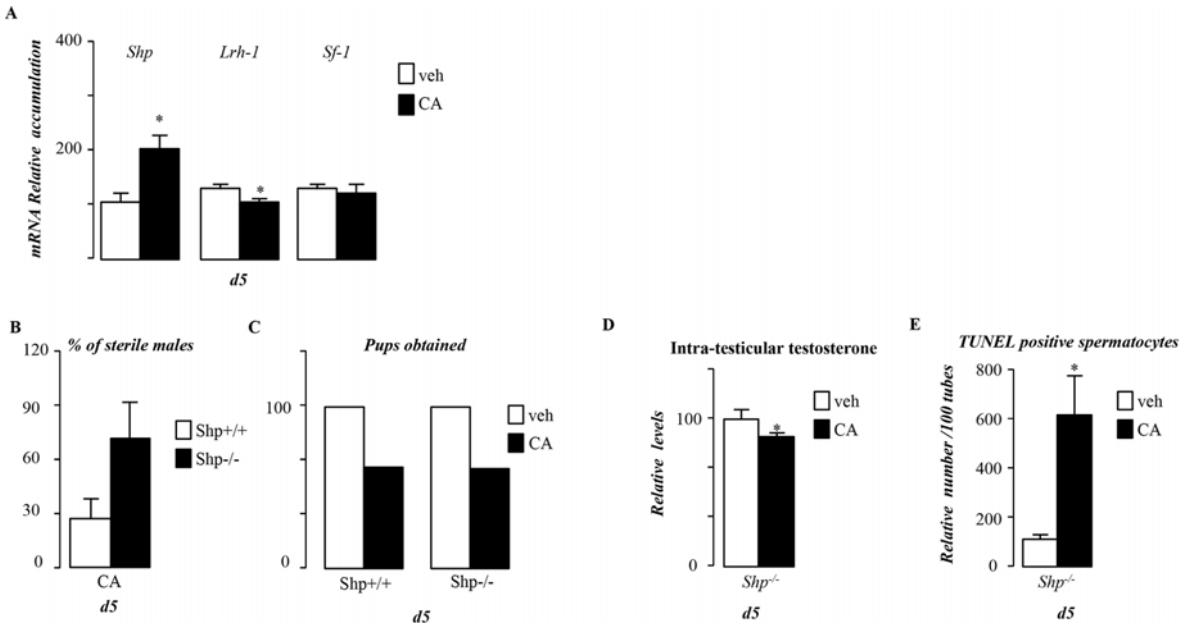

Figure 6: BA act in though FXRα in a SHP independent manner in pubertal males. A. Testicular mRNA expression of *Shp, Lrh-1*and *Sf-1* normalized to *β-actin* levels in whole testis of C57Bl/6J mice fed control or CA diets for 5 days (n=5 to 10 per group). **B.** Percentage of infertile males in Shp+/+ and Shp-/- exposed to CA diet for 44 days. **C.** Total number of pups obtained per group in Shp+/+ and Shp-/- exposed to control or CA diet for 44 days. **D.** Relative intra-testicular testosterone levels in Shp-/- mice fed control or CA diet for 5 days (n=6-10 per group) **E.** Quantification of TUNEL analyses in Shp-/- males fed control or CA diets for 5 days. The number of TUNEL-positive spermatocytes is indicated as the number of positive cells per 100 seminiferous tubules (n=6-10). In all of the panels data are expressed as the means ± SEM. Statistical analysis:*, $p<0.05$; **, $p<0.01$, ***, $p<0.005$ *vs.*respective control group.

Interestingly, the lower sensitivity to LH/hCG induced by GW4064 was not observed in Fxrα-/- males as revealed by the measurements of testosterone levels (Figure 8G), *Star* mRNA accumulation (Figure 8H).

Consistent with *in vivo* experiments, pre-treatment of MA10 cells with GW4064 for 12 hours decreased the response to hCG with lower sensitivity regarding steroid production in GW4064 condition compare to vehicle treated cells (Figure 9A). This was supported by the lower fold-induction of steroidogenic genes such as *Star* in GW4064 condition compare to vehicle treated cells (Figure 9B). The altered sensitivity of MA10 cells to LH/CG following GW4064 treatment, was supported at the level of the intracellular signaling as measured by the lower increased of CREB phosphorylation in GW4064 treated compare to vehicle group (Figure 9C).

The involvement of the down-regulation of the *Lhcgr* expression in these effects was sustained by the fact that GW4064 was not able to counteract nor the effect of Forskolin (Figure 9D), an adenylate cyclase activator, neither the impact of cAMP analog 8-BromocAMP (Figure 9D). Interestingly, the expression of *Dax-1* was demonstrated to be inhibited by LH/hCG pathway. If GW4064 counteracted the effect of LH/hCG on *Dax-1* mRNA accumulation, it had no effect on the impact of FSK and 8Bromo-cAMP on Dax-1 (Supplementary Figure S4D).

In order to analyze the potential individual or combined roles of DAX-1 and SHP in this lower sensitivity

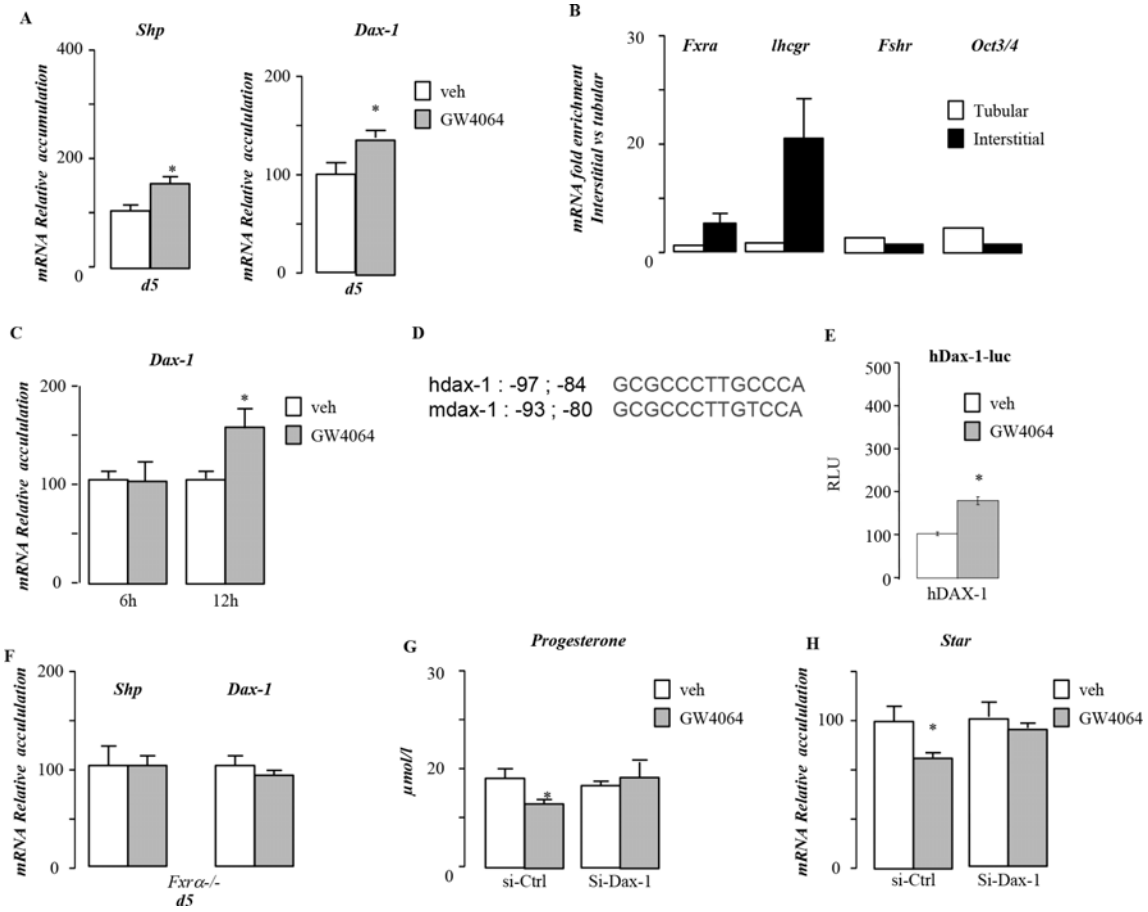

Figure 7: Dax-1 is a target gene of FXRα. A. Testicular mRNA expression of *Shp* and *Dax-1* normalized to *b-actin* levels in whole testis of C57Bl/6J mice treated with vehicle or GW4064 for 5 days (n=5 to 10 per group). **B.** mRNA expression of *Fxrα*, *Lhcgr*, *Fshr* and *Oct3/4* normalized to *β-actin* levels in interstitial and tubular compartment of 15-days-old testis of C57Bl/6J. **C.** mRNA expression of *Dax-1* normalized to *β-actin* levels in MA-10 cells treated for 6hours or 12 hours with vehicle or GW4064 (n=10 per group). **D.** Sequences of FXRE putative biding site (IR1) in human and mouse DAX-1 promoters. **E.** CV1cells were transfected with pCMX-mFXRα, pCMX-mRXRα, or both receptor plasmids in the presence of the 1.2kbDAX-1 promoter (hDAX1) linked to luciferase. Cells were treated with GW4064 (1 µM). **F.** Testicular mRNA expression of *Shp* and *Dax-1* normalized to *β-actin* levels in whole testis of *Fxrα-/-* mice treated with vehicle or GW4064 for 5 days (n=5 to 10 per group). **G.** Relative progesterone levels in medium of MA10 cells transfected with siRNA-control or siRNA directed against Dax-1 and treated for 12 hours with vehicle or GW4064 (n=6-15 per group). **H.** mRNA expression of *Star* normalized to *β-actin* levels in MA10 cells transfected with siRNA-control or siRNA directed against Dax-1 and treated for 12 hours with vehicle or GW4064 (n=10 to 15 per group). In all of the panels data are expressed as the means ± SEM. Statistical analysis:*, $p<0.05$; **, $p<0.01$, ***, $p<0.005$ *vs.*respective control group.

to LH/hCG signaling, we performed experiments using MA-10 cell lines. The decreased sensitivity to LH/hCG was observed after 6h or 12h after GW4064 exposure (Figure 9F). However, the increase of *Dax-1* mRNA accumulations in response to GW4064 treatment was only observed at 12h (Figure 7C) These results support the idea that neither SHP nor DAX-1 were main mediators of the effects of the FXRα-GW4064 effects on LH/hCG stimulated steroidogenesis within Leydig cells. We next tested the impact of GW4064 on known repressor of Lhcgr gene. We tested the expression of Ear2 and Couptf. In vivo data did not allowed determining the molecular mechanisms how FXRα could inhibit Lhcgr expression as Ear2 was not detected on prepubertal testis and Couptf expression was not affected by GW4064 treatment (data not shown).

Finally, we wondered whether this effect of FXRα signaling pathway on LH/hCG stimulated steroidogenesis was specific to pubertal age. In order to answer such question, 12day-old mice were exposed for 5 days to GW4064. At this age, no impact on testosterone levels (Supplementary Figure S5A) or apoptotic germ cell rate was observed in response to GW4064 compare to vehicle-treated group (Supplementary Figure S5B). In addition, the analyses showed that at this age GW4064 altered the sensitivity to LH/hCG. Indeed, chow-treated group showed a 8.6 fold increase of testosterone levels in response to LH/hCG whereas

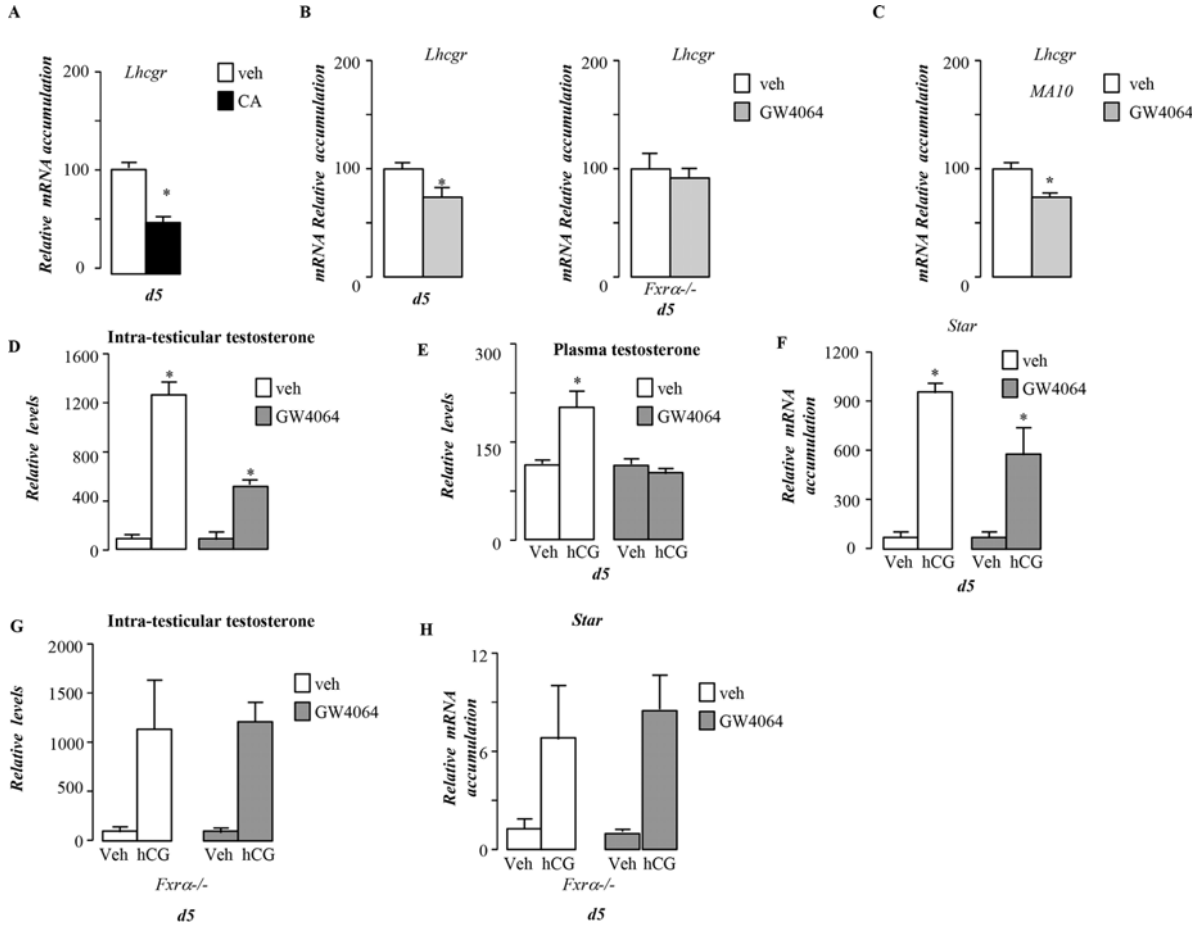

Figure 8: BA signaling pathway alters LH/CG sensitivity in pubertal males. A. Testicular mRNA expression of *Lhcgr* normalized to *β-actin* levels in whole testis of C57Bl/6J mice fed control or CA diets for 5 days (n=5 to 10 per group). **B.** Testicular mRNA expression of *Lhcgr* normalized to *β-actin* levels in whole testis of C57Bl/6J or *Fxrα*-/- mice exposed to vehicle or GW4064 for 5 days (n=5 to 10 per group). **C.** mRNA expression of *Lhcgr* normalized to *β-actin* levels in MA-10 cells treated for 6 hours with vehicle or GW4064 (n=10 per group). **D.** Relative intra-testicular testosterone levels in C57Bl/6J mice treated 5 days with vehicle orGW4064 and then 12 hours with vehicle or hCG (n=5-15 per group). **E.** Relative plasma testosterone levels in C57Bl/6J mice treated 5 days with vehicle or GW4064 and then 12 hours with veh or hCG (n=5-15 per group). **F.** Testicular mRNA expression of *Star* normalized to *β-actin* levels in whole testis of C57Bl/6J mice treated 5 days with vehicle or GW4064 and then 12hours with veh or hCG. **G.** Relative intra-testicular testosterone levels in *Fxrα*-/- mice treated 5 days with vehicle, GW4064 and then 12 hours with vehicle or hCG (n=5-15 per group). **H.** Testicular mRNA expression of *Star* normalized to *β-actin* levels in whole testis of *Fxrα*-/- mice treated 5 days with vehicle or GW4064 and then 12hours with veh or hCG. In all of the panels data are expressed as the means ± SEM. Statistical analysis:*, $p<0.05$; **, $p<0.01$, ***, $p<0.005$ *vs.*respective control group.

GW4064-treated mice presented only a 2 fold increase (Supplementary Figure S5C). In contrast, at 44-dpt, if the impact of CA-diet on testosterone levels still present, the sensitivity to LH/hCG was no more present on CA-diet exposed mice (Supplementary Figure S5D).

DISCUSSION

In the present work, we point out that BAs alter testicular physiology during sexual maturation. We demonstrate that BA exposure could act on testis endocrine function. We provide evidence for the critical roles played by the FXRα-SHP/DAX1 signaling pathways in the regulation of basal steroidogenesis (Figure 10). Moreover, we showed that BA signaling alter the response to the hypothalamo-pituitary axis, the main regulator of endocrine function (Figure 10). This effect seems to be due to the control of the LH receptor expression by FXRα.

One could have thought that systemic effect could be involved on the described phenotype following GW4064 or CA-diet. In similar way of the intestine/liver pathway, the role of the FGFR4/β Klotho signaling, previously described as a target of FXRα could be hypothesized as a regulator of the testicular FXRα pathways as FGF signaling is known to regulate testicular physiology. However, in testis this signaling relies on paracrine interactions between Leydig and Sertoli cells. Indeed, if Fgfr4 is expressed in Leydig cells and regulates steroidogenesis, the FGF ligands are expressed and secreted by the Sertoli cells. Thus regarding the present study as the effects of GW4064 on testicular steroidogenesis as well as on sensitivity to LH/hCG

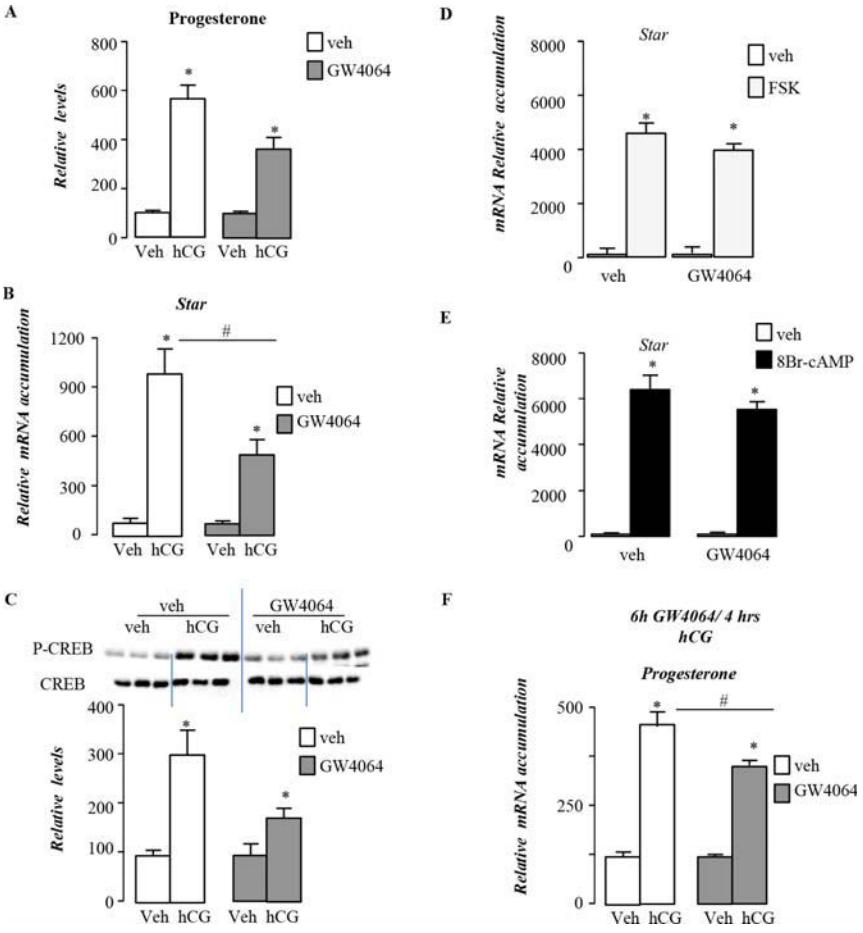

Figure 9: BA signaling pathway alters LH/CG sensitivity at the Leydig cell level in pubertal males. A. Relative progesterone levels in medium of MA10 cells pre-treated 12h with vehicle or GW4064 and then 4 hours with veh or hCG (n=5-15 per group). **B.** mRNA expression of *Star* normalized to *β-actin* levels in MA10 cells pre-treated 12h with vehicle or GW4064 and then 4 hours with veh or hCG (n=5-15 per group). **C.** Representative western bots of P-CREB and CREB, and quantification of the P-CREB/CREB ratio in MA10 cells MA10 cells pre-treated 12h with vehicle or GW4064 and then 30min with veh or hCG (n=5-15 per group). **D.** mRNA expression of *Star* normalized to *β-actin* levels in MA10 cells pre-treated 12h with vehicle or GW4064 and then 4 hours with veh or Fsk (n=5-15 per group). **E.** mRNA expression of *Star* normalized to *β-actin* levels in MA10 cells pre-treated 12h with vehicle or GW4064 and then 4 hours with veh or 8Bromo-AMPc (n=5-15 per group). **F.** Relative progesterone levels in medium of MA10 cells pre-treated 6h with vehicle or GW4064 and then 4 hours with veh or hCG (n=5-10 per group). In all panels data are expressed as the means ± SEM. Statistical analysis:*, $p<0.05$; **, $p<0.01$, ***, $p<0.005$ *vs.*respective control group.

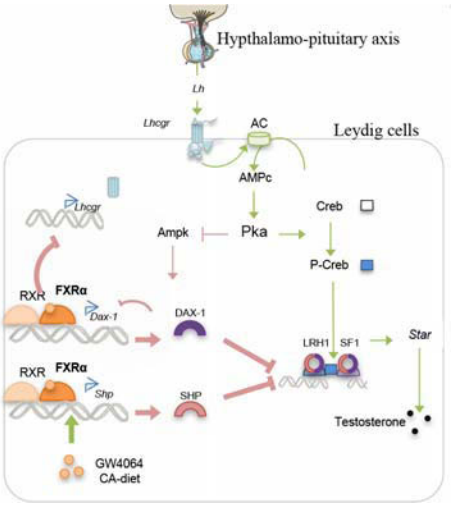

Figure 10: Proposed model for the action of BA-FXRα pathways on Leydig cells during puberty.

were reproduced on the Leydig MA-10 cell line, it does not support the idea of the involvement of the paracrine FGF-FGFR4 pathway. In addition the expression of Fgfr4 was not altered by the treatment with GW4064 (data not shown). Moreover, we were not able to detect bKlotho in pubertal testis.

Thus, the present study suggests that BA signaling pathways involved might be different between pubertal and adult male mice. Indeed, we have previously shown that BA-exposure in adult leads to infertility. We demonstrate that these effects are strictly mediated by TGR5 [11]. However, it has to be noticed that the short-term impact of BA-diet on steroidogenesis had not been fully studied in adult mice [11]. Thus we could not exclude that such phenomena could also happened in adult mice. It is interesting to note that for other kinds of exposure such as for endocrine disrupters, the window of exposure is critical in regards of the observed phenotypes. Indeed, in utero/neonatal exposure leads to infertility [10] whereas adult exposure leads to transitory germ cell apoptosis [15].

Surprisingly, the invalidation of Shp was not sufficient to avoid the impact of BA exposure on testis physiology. Indeed, if we previously identified that, in adult mice, very short-term exposure to FXRα agonist represses testosterone synthesis *via* SHP, the present work suggests that in longer exposure (chronic exposure, 5-days); FXRα could represses steroidogenesis in a SHP-independent manner. This must be explained by our results demonstrating for the first time that *Dax-1*, a well-known repressor of steroidogenesis within Leydig cells [12], is a direct transcriptional target gene of FXRα. Our data suggest that Dax-1 is involved in the basal repression of steroidogenesis by FXRα. This suggest a first mechanism how FXRα represses steroidogenesis, through the elevation of *Dax-1* and *Shp* expression and their subsequent repressive effects on transcription. Indeed, these two receptors are known repressors of steroidogenesis *via* the interaction with SF1 and/or LRH-1. Interestingly, Dax-1 was previously demonstrated as a target of SF-1 which might be a feedback loop to regulate steroidogenesis. However, in our study, no effect of FXRα signaling was observed on Sf-1 expression. Interestingly, SF-1 and LRH-1 are known to use same response element in sequence of target genes thus dax-1 could be a LRH-1 target gene. However, FXRα represses the expression of Lrh-1, thus it does not feet with induction of Dax-1. We demonstrate here that FXRα directly induces the expression of *Dax-1*. In addition, the regulation of Dax-1 is of interest as it has been recently demonstrated that DAX-1 acts as a co-repressor of FXRα through the competition with co-activators such as SRC-1 and PGC-1α [16]. Combined, these data suggest the existence of a potential negative feedback for a local control of steroidogenesis. This also opens new interesting field of research as *Dax-1* expression is associated with altered reproductive function.

The potential redundancy between SHP and DAX-1 is supported by the fact that even if SHP participates to the response of testicular physiology in case of low LH/CG levels [17], $Shp^{-/-}$ males present normal level of LH and normal answer to acute LH surge [21].

The data reported here suggest that the FXRα signaling pathway might also be a regulator of the LH/hCG stimulated testicular steroidogenesis. Indeed, the main striking point of the present work is the identification of the molecular mechanisms of the interaction between FXRα signaling pathway and the hypothalamo-pituitary axis. Our data supported the evidence that the gene encoding the LH-receptor is repressed by FXRα pathways. This demonstrates that BA signaling pathway, at the testicular level, interact with the hypothalamo-pituitary axis in the regulation of testosterone production. This is of importance as, the control of the Leydig cell functions, including steroidogenesis, is predominantly mediated by

the hypothalamo-pituitary-gonadal axis via LH/CG [7], which is of major importance for the initiation of sexual maturation.

The interaction of the two defined mechanisms is reinforced by the fact that some impact of LH signaling have been described to be dependent of LRH-1/SF-1 binding sites on the regulatory sequences of steroidogenic genes such as Star [18, 19]. Moreover, there might be a cross-talk between the regulation of *Shp* and *Dax-1* with the altered LH signaling in response to BA as these two targets of FXRα have been shown to be negatively regulated by the LH pathways *via* PKA-AMPK pathways [17, 20].

A role of FXRα in Leydig cells, a question of age? At 12-day-old, FXRα mainly impacts the sensitivity of Leydig cells to LH/hCG, at 20-day-old its signaling alters both basal and LH/hCG stimulated steroidogenesis, and finally at 44dpt the FXRα signaling pathway seems to impact only basal steroidogenesis. Such differences regarding the effects of FXRα on Leydig cell functions could be the results of the regulation of particular target genes. Regarding basal steroidogenesis, either Dax-1 or Shp could be involved. Interestingly, it was previously demonstrated that SHP is expressed in the Leydig cells only since 20-day-old [1]. This could explain in part that FXRα have almost no impact on basal steroidogenesis before 20-day-old.

Interestingly, if FXRα affects LH/hCG sensitivity at 12 days-old, it has only slight impact of basal testosterone levels which was consistent with the lack of effects on germ cell apoptosis rate. These data suggest that at this age the unidentified repressor of Lhcgr in response to activation of FXRα might be induced whereas Dax-1 or Shp might not be induced. Regarding SHP this could be consistent with the fact that at this age SHP is not or low expressed in interstitial space at this age [1].

For instance, we have no clue to explain the impact of FXRα on *Lhcgr* expression as we did not identified the involved repressor. However, our results suggest that the actor(s) invoved in the downregulation of *Lhcgr* in response to FXRα activation could be with a limited expression during prepubertal age.

The present work opens new field of research to better understand physiological and pathological conditions. The remaining question, which will require additional studies, will be to clearly decipher the physiological role of FXRα and BA within Leydig cells. We need to determine in which conditions the FXRα signaling pathway participate to the repression of Leydig steroidogenesis. Several hypotheses could be made. The first point will be to define the endogenous ligand of FXRα within the testis. We have demonstrated that BA are present in the testis in normal conditions [11]. In addition, others defined that steroids derived from androgen catabolism, like androsterone, are potential FXRα ligands [21]. In that line we can make the hypothesis of a testicular feedback loop to repress androgen production as a negative feedback [22]. Combined these data suggest that FXRα could be activated by numerous stimuli within the testis.

Our data reinforce the links between FXRα signaling pathways and steroid metabolism [23, 24]. This is of major importance as endocrine homeostasis is a critical physiological process, as alteration could lead to various diseases. This point has been enlightened in the last decades with the large impact of endocrine disrupters on animal and human health [10, 25]. We thus define FXRα as an important actor of the regulation of testicular physiology during puberty. This is of interest in order to identify the etiology of primary hypogonadism observed in case of liver disorders during puberty period as demonstrated in experimental models [2, 26]. Our results are sustained by the fact that impaired growth and delayed puberty are found in case of progressive familial intrahepatic cholestasis [27]. Combined, the present data and other work [11]support the physiological roles of BA signaling pathways on testicular physiology at different timing during male life.

MATERIALS AND METHODS

Ethics statement

This study was conducted in accordance with the current regulations and standards approved by the Animal Care Committee (CEMEA Auvergne) (CE-60-12).

Animals

C57Bl/6J were purchased from Charles River Laboratories (L'Arbresle, France); *Shp*$^{-/-}$ Fxrα-/- and *Tgr5*$^{-/-}$ mice have been previously described [11, 10, 28, 29]. The mice used in this study were maintained on a C57BL/6J background and housed in temperature-controlled rooms with 12 hours light/dark cycles. Mice had *ad libitum* access to food and water. 21-days old mice were fed to D04 diet (control) or D04 diet supplemented with 0.5% cholic acid (CA-diet) (SAFE, Augy, France) for 5, 14, 26 or 44 days. As young mice are quite sensitive to CA-diet, they were fed 5 days with CA-diet and 2 following days with the control diet. This sequence was repeated until sacrifice. The choice of this age was to be more efficient in the treatment with CA-diet since from 19 day-old, pups are separated from the mother and thus get fed only by diet. Moreover, it allows to target particular window of the increase of testicular to reach adult levels.

The fertility tests were performed at 44 days after the beginning of the treatment.

For GW4064 experiments, 21-days old mice were daily injected (intra-peritoneal) with 37µl of vehicle (DMSO) or GW4064 (28mg/kg) during 5 days. For specific experiments, mice were injected with hCG (5 IU, equivalent to 1.42mM; Sigma-Aldrich) diluted in NaCl 0.09%.

Sperm count

The epididymis was harvested. Then the head or tail was mashed and we count head of spermatozoa in order to reflect the sperm production.

Histology

Testes were collected, paraformaldehyde (PFA)-fixed and embedded in paraffin, and 5 µm-thick sections were prepared and stained with hematoxylin/eosin.

TUNEL analysis

TUNEL experiments were performed as previously described on 5 µm of testis fixed in PFA 4% [5]. In each testis, at least 100 random seminiferous tubules were counted. Results are expressed as the percentage of tubules with either spermatocytes or spermatids TUNEL-positive.

Endocrine investigations

Steroids were extracted from testes as previously described [5]. Intra-testicular and plasma levels were measured using commercial kits: testosterone and estradiol (Diagnostic Biochem, London, Canada).

Real-Time RT-PCR

RNA from testis samples were isolated using Nucleospin RNA (Macherey-nagel, Hoerdt, France). cDNA were synthesized from total RNA with the MMLV reverse transcriptase and random hexamer primers (Promega, Charbonnière Les Bains, France). Real-time PCR measurement of individual cDNAs was performed using SYBR green dye (Master mix Plus for SYBR Assay, Eurogentec, Angers, France) to measure duplex DNA formation with the Eppendorf Realplex system. Sequences of primers are reported in Supplementary Table S1. Standard curves were generated with pools of testis cDNA from animals with different genotypes and/or treatments. Results were analyzed using the ΔΔct method.

Cotransfection assays

CV1 cells were transfected as described [14]. hDAX-1 promoter-luciferase reporter [30] was kindly provided by Dr E. Lalli. h-NR0B-1-luc construct (50 ng) was added in combination with CMX-mFXRα (15 ng), CMX-mRXRα (15 ng), β-galactosidase (10 ng), and pCMX for a total of 150 ng/well. Ligands were added 6-8 hours later in serum free media. Cells were harvested 14-16 hours later and assayed for luciferase and β-galactosidase activity. Luciferase values were normalized for transfection efficiency using β-galactosidase and expressed as RLU of triplicate assays (mean ± SD).

siRNA transient transfection

MA10 cells were transfected with small interfering RNA (siRNA) using interferin (Ozyme, Saint Quentin Yvelines, France) in six-well plates (400,000 cells per well). The siRNA directed against *Dax-1*, as well as control siRNA (siGfp), was transfected at 5 ng per well. When 48 hours after the transfection had passed, cells were treated with vehicle (DMSO, 1/1,000) or GW4064. Then, cells were harvested 12 hours later, and mRNA extractions were performed.

Electrophoretic mobility shift assays

EMSAs were performed as previously described [14].

Experiments were done in vitro translated proteins for FXRα and RXR using the appropriate labeled probe (IR1-hDAX1:5′-CCGCGCCCTTGCCCAGACCGAGGCG-3′). Specificity RXR-FXR of binding was tested by competition with × 100, × 50, and × 20 excess of various unlabeled FXREs (IR1-hFGF19 [13]) or LXRE (LXRE-abca1 [14]); After electrophoresis, gel was dried at 80°C for 1 h and autoradiographed with intensifying screen at −80°C overnight.

Cell culture experiments

MA10 cells were maintained at 37°C in an atmosphere of 5% CO_2 with Waymouth (Life Technologies) containing 100 U/ml penicillin and 100 µg/ml streptomycin supplemented with 10% horse serum. On d0, MA10 cells were seeded at 400 × 10^3 cells per well in 6-well plates and allowed to adhere overnight. The following day, cells were washed twice with 1 × PBS, and the medium without serum was applied with the GW4064 (10^{-6}M) or vehicle (DMSO). In some experiments, cells were then treated with vehicle (Nacl) or hCG (2.5nM), Forskolin (10 µM) or 8BrAMPc (100 µM) for 4 hours.

Statistics

Differences between *two groups* for single point data were determined by Student's *t*-test. For other data obtained two-way analysis of variance was performed. When significant effects were obtained, multiple comparisons were made with Tukey's test. All numerical data are represented as mean ± SE. Significant difference was set at $P< 0.05$.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

Authors' contribution

MB, EM, AV, LS, BR: performed experiments

MB, EM, SB, KS, FC, DV: participate to discussion of results and conception of experiments

DHV write the manuscript

All authors participate to the corrections of the manuscript.

Abbreviations

AMPK, AMP activated protein kinase; BA, bile acid; BTB, blood-testicular barrier; CA, cholic acid; cDNA, complementary DNA; Cyp3a25, cytochrome P450, family 3, subfamily a, polypeptide 25; Dax-1, dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene-1, FXR-α, farnesol X receptor; GPBAR1, G-protein-coupled bile acid receptor 1; hCG, human choriogonadotropin; H& E, hematoxylin and eosin; IHC, immunohistochemistry; IP, intraperitoneal; IT, intratesticular; LH, luteinizing hormone; mRNA,; LRH-1: liver receptor homolog-1; messenger RNA; PFA, para-formaldehyde; PKA, Protein Kinase A; SF-1; Steroidogenic factor-1; SHP: Small heterodimer partner; Star, steroidogenic acute regulatory protein; Sult2a1, sulfotransferase family, cytosolic, 2A, TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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Discussion et perspectives de l'article II

Les résultats de ce travail démontrent que la sur-activation de la signalisation associée à Fxrα chez des animaux pré-pubères de 21 jours, entraîne un défaut développemental du tractus génital (testicules, épididymes, Vésicules Séminales) indépendant de la testostérone plasmatique, dont la concentration n'est pas altérée (données non montrées). Elle se traduit par ailleurs précocement par un retard de la différenciation post-méiotique des cellules germinales. Cette altération est associée à une apoptose transitoire de la lignée germinale, spécifique du stade spermatocyte, due à une hypo-androgénie testiculaire, et abouti à un phénotype d'infertilité à l'âge adulte.

D'un point de vue moléculaire, nous identifions le récepteur nucléaire Dax-1, un répresseur connu de l'expression des enzymes stéroïdogènes, comme un nouveau gène cible direct de Fxrα, et suggérons qu'il soit un nouvel acteur impliqué dans la répression de l'activité endocrine des cellules de Leydig par Fxrα. Enfin, nos données mettent en évidence pour la première fois l'existence d'une interaction entre les voies de signalisation associées à Fxrα et à l'hormone Lh.

1. La répression de la stéroïdogenèse en réponse au régime CA et au traitement GW4064, est-elle le résultat de l'activation de la signalisation associée à Fxrα ?

Nous montrons que la répression de la stéroïdogenèse, traduite par une diminution de l'accumulation des transcrits de Star, observée *in vivo* en réponse au régime CA, est reproduite par l'utilisation du GW4604, un agoniste spécifique de Fxrα. Ces données suggèrent que cette altération puisse être le résultat de l'activation de la signalisation associée à Fxrα. Cependant, de nouvelles expériences devraient être conduites afin d'apporter la preuve formelle de l'implication de ce récepteur dans l'effet répresseur du CA et du GW4064 sur l'activité endocrine des cellules de Leydig :

1- des animaux sauvages et Fxrα$^{-/-}$ seront soumis à un régime CA, ou au GW4064 pour une durée de 5 jours ;

2- des cellules MA10 seront traitées avec du GW4064 pour une durée de 12 h, en présence d'un siRNA dirigé spécifiquement contre Fxrα.

Dans ces deux modèles complémentaires, *nous analyserons le niveau d'expression du gène codant l'enzyme Star, et doserons la testostérone intra-testiculaire (modèle in vivo), et la progestérone sécrétée dans le milieu de culture (cellules MA10).*

2. Shp et Dax-1 : deux acteurs redondants pour la répression de la stéroïdogenèse induite par la signalisation associée à Fxrα ?

Dans notre modèle d'exposition pré-pubertaire au GW4064, nous montrons que l'activation supposée de Fxrα réprime la stéroïdogenèse par des mécanismes indépendants de Shp, contrairement à ce qui a été démontré en 2007 chez l'adulte.

a) Dax-1, le lien entre Fxrα et répression de la stéroïdogenèse ?

Nous montrons que l'accumulation des transcrits de Dax-1 est augmentée *in vivo* et *in vitro* en réponse au régime CA et au GW4064. L'activation de Dax-1 étant associée à la répression de la stéroïdogenèse (342), il pourrait ainsi participer à l'inhibition de l'expression de Star induite par Fxrα. *Cette hypothèse devrait être testée par l'utilisation d'un siRNA dirigé contre Dax-1 dans le modèle cellulaire MA10 traité au GW4064 pour une durée de 12 heures. L'expression du gène codant Star sera alors analysée par RT-qPCR.*

b) Quelles hypothèses pour expliquer cette discordance Shp-Dax-1 ?

1- La redondance de Shp et Dax-1 : les récepteurs nucléaires Shp et Dax-1 ont une action redondante, ainsi Dax-1 pourrait-il compenser la perte de Shp. Cette hypothèse semble peu probable au vu du profil d'expression des gènes impliqués dans la synthèse des stéroïdes sexuels chez les modèles murins dont le gène codant Shp ou Dax-1 a été invalidé. En effet, les animaux $Shp^{-/-}$ présentent une augmentation de l'accumulation des transcrits des gènes codant les enzymes stéroïdogènes Star, Cyp11a1 et 3β-Hsd, tandis que celle du gène codant l'aromatase semble être diminuée (données préliminaires non publiées) (33). A l'inverse l'expression de l'aromatase est augmentée chez les souris Dax-$1^{-/-}$, et restaurée par la surexpression de Dax-1 spécifiquement dans les cellules de Leydig ; de plus, l'expression de Star, Cyp11a1 et 3βHsd n'est pas altérée dans ces deux lignées murines transgéniques pour Dax-1 par rapport aux animaux sauvages (399)-(400).

2- La durée du traitement : 5 jours dans notre modèle, 12 heures pour les travaux de Volle *et al*. (2007). Shp pourrait ainsi être impliqué dans les effets aigus du GW4064, tandis que Dax-1 prendrait le relai pour les effets chroniques. *Cette hypothèse pourrait être approchée par de nouvelles cinétiques d'exposition d'animaux pré-pubères au GW4064 : une exposition de* ***12 heures*** *chez des animaux sauvages et* ***Shp$^{-/-}$****, et une exposition de* ***5 jours*** *chez des animaux ayant subi une injection intra-testiculaire de* ***siRNA dirigé contre Dax-1****.*

3- Le statut de maturation des cellules de Leydig : dans notre modèle, les souris sont exposées de 21 à 26 jours post-nataux, période au cours de laquelle les cellules de Leydig subissent un processus de maturation et de prolifération. Chez l'adulte, ces cellules sont quiescentes et complètement matures. Ces deux populations de cellules de Leydig expriment sans doute un panel de gènes différent, qui pourrait expliquer la différence de signalisation mise en jeu chez les animaux

pré-pubères et adultes, en réponse à l'activation de Fxrα. De façon intéressante, Shp est faiblement détecté dans le compartiment interstitiel au cours du développement post-natal précoce, et son expression augmente graduellement à partir du 25ème jour post natal jusqu'à l'âge adulte (33). Shp est donc peu exprimé au cours de notre fenêtre d'exposition au régime CA et au GW4064. Qu'en est-il du profil d'expression de Dax-1 dans les cellules de Leydig au cours du temps ? *Une telle analyse serait interessante afin de déterminer si il peut être inverse à celui de Shp. Nous nous proposons par ailleurs d'exposer au GW4064 des animaux* ***adultes sauvages et Shp$^{-/-}$*** *pour une durée de* ***5 jours****, ainsi que des animaux pré-pubères et adultes ayant subi une injection de siRNA dirigé contre Dax-1 afin de tester la véracité de cette hypothèse.*

L'analyse de l'expression du gène codant Star, et le dosage de la testostérone intra-testiculaire (modèle in vivo), et la progestérone sécrétée dans le milieu de culture (modèle in vitro) permettront de valider ou non ces 2 dernières hypothèses.

3. L'inhibition de l'expression des gènes codant les enzymes stéroïdogènes est-elle la seule responsable de la répression de la synthèse d'androgènes ?

La diminution de la concentration intra-testiculaire de testostérone, observée *in vivo* après 5 jours de régime CA ou de traitement GW4064, est le résultat d'une répression du gène codant l'enzyme limitante de la stéroïdogenèse Star, ainsi que l'enzyme Cyp11a1 (données non montrées) en réponse à l'activation supposée de la signalisation Fxrα. Cependant, nous ne pouvons exclure un impact sur la disponibilité du substrat de la stéroïdogenèse : le cholestérol. En effet, les récepteurs Sf-1 et Lrh-1 sont connus pour activer l'expression des gènes codant Sr-b1 et Hmg-CoA synthase et réductase, impliqués respectivement dans le captage et la biosynthèse du cholestérol. Ainsi l'activation de Dax1 et / ou de Shp par le régime CA et le traitement GW4064, pourrait-elle conduire à l'inhibition de l'activité transcriptionnelle de Sf-1 et Lrh-1 sur le promoteur de ces 2 gènes, et par conséquence à une diminution de la concentration intra-testiculaire de cholestérol. Nous pourrions aborder cette question en *examinant le profil d'expression de Sr-b1 et Hmg-CoA synthase et réductase in vivo et in vitro en réponse au CA et au GW4064, et doser le cholestérol et les esters de cholestérol dans les cellules MA10 traitées au GW4064.* Des données préliminaires montrent que l'expression de Sr-b1 et Hmg-CoA réductase est diminuée dans les cellules MA10 après 12 et 24 heures de traitement au GW4064, soutenant cette hypothèse.

4. Par quels mécanismes le GW4064 diminue-t-il la sensibilité des cellules de Leydig à la Lh ?

Le pré-traitement au GW4064 diminue *in vivo* et *in vitro* la sensibilité des cellules de Leydig à la Lh / hCG. Cet effet est annulé lorsqu'*in vitro* l'hCG est remplacée par la forskoline ou le 8-Bromo-AMPc, démontrant que l'effet du GW4064 est centré sur le récepteur à la Lh. L'expression de ce dernier est en effet diminuée après un traitement au GW4064 de 5 jours in vivo, et de 6 heures *in vitro*.

a) L'effet du GW4064 est-il spécifique de Fxrα ?

Des données préliminaires montrent que le pré-traitement de cellules MA10 avec l'INT-747, un autre agoniste synthétique de Fxrα, reproduit la diminution de l'induction de l'expression de Star en réponse à l'hCG, observée avec le GW4064. Ces résultats soutiennent l'implication de Fxrα dans l'interaction du GW4064 avec la voie de signalisation associée à la Lh. Afin de s'en assurer, de nouvelles expériences devraient être menées *in vivo* et *in vitro* :

1- analyser l'expression du récepteur à la Lh chez des animaux sauvages et Fxrα$^{-/-}$ après 5 jours de régime CA et / ou de traitement GW4064, et dans les cellules MA10 traitées pendant 6 heures au GW4064 en présence ou non d'un siRNA dirigé contre Fxrα ;

2- des animaux sauvages et Fxrα$^{-/-}$ devraient subir un pré-traitement de 5 jours au GW4064 avant une injection d'hCG ;

3- des cellules MA10 devraient être pré-traitées pendant 12h avec du GW4064 avant un traitement à l'hCG de 4 heures, en présence ou non d'un siRNA dirigé contre Fxrα.

Nous analyserons dans ces deux derniers modèles in vivo et in vitro de pré-traitement au GW4064 la réponse à l'hCG : le niveau d'expression du gène codant Star, et le dosage de la testostérone intra-testiculaire et de la progestérone sécrétée dans le milieu de culture.

b) Par quels mécanismes Fxrα diminue-t-il la transcription du gène codant le récepteur à la Lh ?

1- Dax-1 : des données préliminaires *in vitro* montrent qu'après 6 heures de traitement au GW4064 l'accumulation des messagers du récepteur à la Lh est diminuée, alors que l'expression de Dax-1 n'est pas encore augmentée. Ces résultats écarteraient l'implication de Dax-1 dans le contrôle de l'expression du gène codant le récepteur à la Lh.

2- SHP : bien que non essentiel dans le contrôle ''basal'' de la stéroïdogenèse par le GW4064, pourrait-il participer à la répression de l'expression du gène codant pour le récepteur à la Lh ? Cette hypothèse semble compromise car, comme Dax-1, l'expression de Shp n'est pas altérée dans les cellules MA10 après 6 heures de traitement au GW4064. *Nous envisageons de mesurer le niveau*

d'accumulation des messagers de Shp et Dax-1 in vitro après 3 heures de traitement au GW4064 : dans le cas ou celui-ci ne serait pas modulé, nous pourrons exclure définitivement ces deux récepteurs du mécanisme d'action responsable de l'altération de la réponse des cellules de Leydig à l'hCG.

3- Fxrα lui-même : dans le cas où ni Dax-1, ni Shp ne soit impliqué, des analyses seraient menées afin d'établir si le récepteur à la Lh est une cible directe de Fxrα. Fxrα est connu pour réprimer la transcription de gènes cibles par deux mécanismes distincts : en entrant en compétition avec un autre facteur de transcription (Hnf4α, Sf-1) pour leur fixation sur une séquence promotrice commune du gène cible, ou en se fixant sous forme de monomère sur un élément de réponse négatif de type GATCCTTGAXXTXX (12). *Nous utiliserons le logiciel Genomatix afin de réaliser une analyse in silico pour la recherche d'éléments de réponse au récepteur Fxrα (FXREs) de ce type dans les séquences régulatrices du gène codant le récepteur à la Lh. Si tel est le cas, une immunoprécipitation de la chromatine avec un anticorps dirigé contre Fxrα, couplée à des études de promotologie devraient être envisagées.*

4- autres acteurs : *nous analyserons également, en réponse au GW4064, le niveau d'expression des récepteurs nucléaires orphelins ERBA-related gene-2* (Ear2, Nr2f6) *et* Ear3/Coup-tfI, *répresseurs connus de la transcription du gène codant le récepteur de la Lh* (401). Dans l'hypothèse où l'accumulation de l'un et / ou l'autre de ces acteurs soit altérée, *nous procéderons, comme pour le récepteur à la Lh, à la recherche de FXREs dans leur séquence promotrice.*

5. Fxrα et catabolisme hépatique de la testostérone ?

Nous avons montré dans l'article I que Fxrα promeut le catabolisme hépatique des androgènes en activant l'expression de gènes codant les enzymes impliquées dans ce processus (Cyp3a11 et Ugt2b34). Le régime enrichi en acide cholique conduit-il chez les animaux pré-pubères, comme chez l'adulte, à l'activation de Fxrα dans le foie, et par ce biais à une augmentation de l'élimination de la testostérone plasmatique ? De cette manière, Fxrα pourrait contribuer au cours du développement pubertaire au maintien d'une concentration intra-testiculaire et plasmatique de testostérone optimale respectivement pour la ''maturation du testicule'', et le développement normal des autres organes du tractus génital (épididymes, vésicules séminales) par le biais de deux mécanismes indépendants : le contrôle de la synthèse des androgènes au niveau testiculaire et de leur catabolisme hépatique. *Le dosage plasmatique de testostérone après 5 et 44 jours de régime CA, corrêlé ou non à l'expression hépatique de Cyp3a11 et Ugt2b34 devrait permettre de valider ou non cette hypothèse.* Des résultats préliminaires montrent qu'après 5 jours de régime CA la concentration plasmatique de testostérone n'est pas altérée. Qu'en est-il à 44 jours de régime ?

6. Fxrα, le lien entre pathologies hépatiques et hypogonadisme primaire ?

Le phénotype testiculaire que nous observons dans notre étude, est similaire à celui détaillé dans les travaux de Van Thiel *et al.* (1985) (397). Ces derniers ont induit une pathologie hépatique chez le rat pré-pubère, qui a eu pour conséquences un blocage de la spermatogenèse et une diminution du nombre de cellules germinales matures, associés à une diminution de la concentration plasmatique de testostérone. Cette atteinte hépatique se traduit notamment par une élévation des niveaux plasmatiques d'acides biliaires. Celle-ci pourrait conduire à l'activation de Fxrα, et ainsi faire le lien entre les pathologies hépatiques et les altérations testiculaires.

Ces travaux ouvrent par ailleurs des perspectives intéressantes chez l'Homme. En effet, la dérégulation de l'expression et / ou de l'activité de FXRα pourrait participer à l'étiologie de défauts pubertaires observés dans plusieurs pathologies, telles que la cholestase intra-hépatique familiale progressive.

Article III

Identification of multiple role of the bile acid nuclear receptor FXRα in mouse testis.

Martinot E, Sedes L, Baptissart M, Rouaisnel B, Saru JP, de Haze A, Thibault-Carpentier C, Keime C, Lobaccaro JMA, Baron S, Benoit G, Schoonjans K, Caira F, Volle DH

En préparation

Les résultats de l'article I et II ont souligné le rôle joué par Fxrα dans le contrôle du métabolisme des androgènes.

Outre son rôle dans le contrôle de l'activité endocrine des cellules de Leydig, l'impact de l'activation *in vivo* de Fxrα sur la physiologie plus globale du testicule n'a jamais été abordé à ce jour. L'objectif du travail présenté dans ce troisième article est donc d'étudier de façon plus complète le rôle potentiel du récepteur Fxrα dans la physiologie du testicule. Nous avons pour cela procédé à l'analyse phénotypique d'un modèle murin dont le gène codant Fxrα a été invalidé.

Identification of multiple roles of the bile acid nuclear receptor FXRα in mouse testis.

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Keywords: Bile acid, FXRα, testis, Spermatogonial stem cells.

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Abstract

The farnesoid-X-receptor (FXRα; NR1H4) is a bile acids (BAs) receptor. Initially, it has been shown to regulate the enterohepatic cycle and the BA biosynthesis. In the last decades, many studies have demonstrated its involvement in other physiological functions (digestion, immunity) and diseases such as diabetes and cancers. FXRα participates to the homeostasis of steroids through the control of either synthesis or catabolism. In the testis, FXRα is expressed within Leydig where it controls testicular testosterone metabolism. However, very few are known about the potential impact of FXRα signaling pathways on testicular physiology. Here using phenotyping approach of FXRα-/- mice, we define new unexpected roles of FXRα within the testis.

Introduction.

The farnesoid-X-receptor (FXRα; NR1H4) is a member of the nuclear receptor family[1,2]. FXRα interacts with the receptor of 9-cis retinoic acid (RXR). FXRα/RXR is a permissive heterodimer, as ligands of both partners can synergize to regulate the transcription of target genes. Bile acids (BAs) were identified as ligands of FXRα. The referred ligands of FXRα are chenodeoxycholic acid (CDCA) and its conjugated derivatives[3]. Individual bile acids have different potencies in regard to the activation of FXRα. The potencies are as follows, in decreasing order: CDCA > DCA (deoxycholic acid) > LCA (lithocholic acid) > CA (cholic acid). FXRα has been demonstrated to play major physiological roles through the use of transgenic rodent models or the use of pharmalogical approaches[4]. Mouse model lacking the gene encoding *Fxrα* (*Fxrα−/−*) highlights its involvement in many physiological functions (digestion, immunity) and diseases such as diabetes and cancers[5]. The first described roles of FXRα were the regulation of the enterohepatic cycle and the regulation of BA biosynthesis[6,7]. *Fxrα−/−* mice exhibit high plasma concentrations of BAs, highlighting the critical role of FXRα in the repression of *Cyp7a1*, which encodes for a key enzyme in BA biosynthesis[8]. It also recently appears that intestinal expression of FXRα is a critical regulator of liver physiology through FGF15/FGF19 signaling pathways[9,10]. FXRα participates in steroid catabolism in the liver and interferes with the steroid signaling pathways in target tissues *via* crosstalk with steroid receptors[3]. In addition, these links with steroid homeostasis was sustained by the identification of the expression of FXRα in the adrenal glands and testes, where it controls steroid

production[11,12]. So far, in the testis, the expression of FXRα within Leydig cells is the only one established, this explain why most of the research were conducted on steroid metabolism[12, 13]. The activation of FXRα during the pubertal period demonstrates the key involvement of its pathways in male sexual maturation through the control of testosterone metabolism (article II). In addition, some recent data clearly suggest that BA exposure alters testicular steroidogenic pathways *via* FXRα even in the context of metabolic syndrome (article annexe VI). These results are of high importance as FXRα is defined as a putative target for treatment of metabolic diseases such as diabetes. All these data suggest that FXRα signaling pathways might play important role within the testis; however, very few are known about the potential impact of FXRα signaling pathways on testicular physiology. Here using phenotyping approach of *Fxrα-/-* mice, we define new roles of this receptor within the testis. Unexpectedly we show here that inactivation of the FXRα signaling pathway resulted in multiple impacts on testicular physiology. Almost all cell types of the testis seem to be affected by the lack of FXRα. This study shows that FXRα define long term reproductive capacity of males. Interestingly, our data provide for the first time evidence that FXRα signaling pathways are involved in defining transcriptome features of progenitor states within the male germline.

Materials and Methods

Ethics Statement. This study was conducted in accordance with the current regulations and standards approved by the Animal Care Committee (CEMEA Auvergne; protocol CE 07-12).

Animals. FXRα-/- mice used have been previously described[14], [15]. Mice used in this study were maintained on housed in temperature-controlled rooms with 12 hours light/dark cycles. Mice had *ad libitum* access to food and water. Nine-week-old mice were fed to A03 diet (SAFE, Augy, France).

Histology. After diet exposure, the testes were collected, formalin-fixed and embedded in paraffin, and 5 µm-thick sections were prepared and stained with hematoxylin/eosin (n=6-10 animals per group).

For the analysis of the blood-testis barrier integrity, 15 µl of EZ-Link Sulfo-NHS-LC-Biotin (7.5 mg/ml) was injected intra-peritoneally (200µl) or an intratesticular (15µl) injection of 0.6 mg of cholyl-lysyl-fluorescein (BD Bioscience, Le Pont de Claix, France)[14]. Thirty min after injection, the testes were harvested, formalin-fixed and embedded in paraffin, and 5 µm-thick

sections were prepared.

TUNEL analysis. TUNEL experiments were performed as previously described[16] on 5 µm of testis fixed in PFA 4%. In each testis, at least 100 random seminiferous tubules were counted. The results are expressed as the number of tubules with either spermatocytes or spermatids TUNEL-positive per 100 seminiferous tubules.

Immunohistochemistry. Paraffin sections of PFA-4% fixed testis were sectioned at 5 µm. The sections were mounted on positively charged glassslides (Superfrost plus), deparaffinized, rehydrated, treated for 20 min at 93–98°C in citric buffer (0.01 M, pH 6), rinsed in osmosed water (2x 5 min) and washed (2 x 5 min) in Tris-buffered saline. Immunohistochemistry was conducted according to the manufacturer's recommendations, as described earlier[17]. Slides were then counterstained with Hoestch medium (1 mg/ml). The antibodies used are given Sox9 [18]; Gata-1[19]; PCNA[17], PLZF[20].

Endocrine Investigations. Testosterone was extracted from testis as previously described[16]. Intra-testicular and plasma testosterone levels were measured using a commercial kit (Diagnostic Biochem, London, Canada).

Bile acid measurements. The BA measurements were performed as previously described[14] and using ELISA assays as recommended by manufacturer (Crystal Chem, Inc. Cat# 80470).

Real-Time RT-PCR. RNA from testis samples was isolated using Nucleospin RNA L (Macherey-nagel, Hoerdt, France). cDNA was synthesized from total RNA with the MMLV reverse transcriptase and random hexamer primers (Promega, Charbonnière Les Bains, France). The real-time PCR measurement of individual cDNAs was performed using SYBR green dye (Master mix Plus for SYBR Assay, Eurogentec, Angers, France) to measure duplex DNA formation with the EppendorfRealplex system. The sequences of primers are reported in [12,14,16,17,21]. Standard curves were generated with pools of testis cDNA from animals with different genotypes and/or treatments. The results were analyzed using the ΔΔct method.

Rnaseq. Rnaseq experiment was performed on testis of wild-type and *Fxrα-/-* mice at 10dpn. Starting from RNA all preparations were made by the IGBMC plateform (Illkirch, France). The mRNAseq libraires were sequenced (1x50b).

Results.

FXRα is expressed in testis since birth. In order to analyze the impact of the nuclear receptor FXRα in testis, we decided to first decipher when it starts to

be expressed in this organ during postnatal period. Using ontogeny approach, Q-PCR data showed that it is expressed from birth to adulthood **(Fig. 1A)**. Its expression seems to decrease from birth to 5-day-posnatal (dpn). From

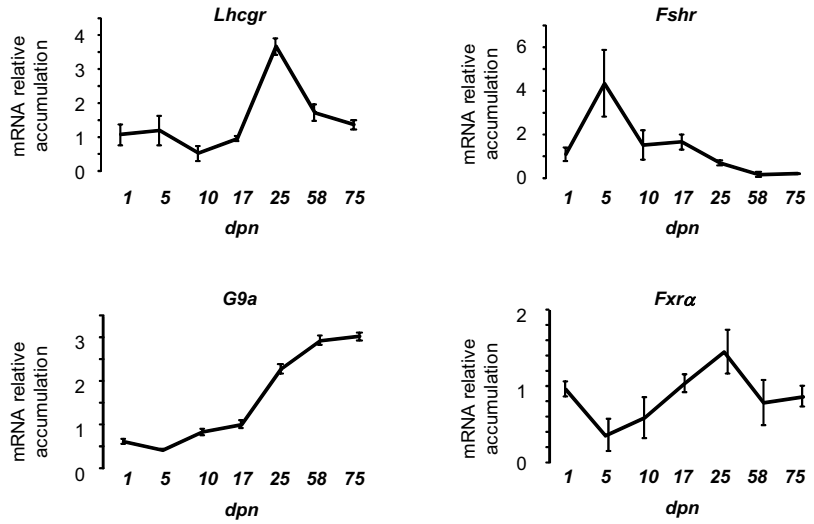

Figure 1. Fxrα is expressed in mice testis. Testicular mRNA accumulation of *Lhcgr*, *Fshr*, *G9a* and *Fxrα* normalized to β–actin mRNA levels in the whole testes of C57BL/6J at 1, 5, 10, 17, 25, 58 and 75 days post-natal. (n=5-10 per age).

5-dpn to 25-dpn *Fxrα* mRNA expression was slightly increased. Thereafter, the expression of *Fxrα* was decreased at the adult age. The profile of the *Fxrα* mRNA accumulation was compared to the ones of specific testicular cell type markers such as *Lhcgr* (Leydig cells), *Fshr* (Sertoli cells) and *G9a* (early germ cell steps).

The results showed that from 25-dpn to adulthood, *Fxrα* showed an expression pattern similar to *Lhcgr* suggesting the expression of Fxrα in Leydig cells at that time. This is consistent with previous studies highlighting its expression in Leydig cells[12,13]. In early postnatal development (from birth to 25-dpn) the expression pattern of *Fxrα* was more similar to the one of *G9a*. This suggests that at this particular time of development, *Fxrα* could be expressed in germ cells. In order to validate these *in vivo* data, we studied the expression of *Fxrα* in testicular mouse cell lines. *Fxrα* mRNA was detected in the adult mouse Leydig cell line MA-10 and in the mouse spermatogonial cell line GC-1spg **(data not shown)**.

FXRα-/- mice show altered testicular phenotype. As *Fxrα* is present in mouse testis from birth; we decided to analyze the impact of *Fxrα* invalidation on testicular phenotype. Surprisingly *Fxrα-/-* males

showed a higher testis weight compared to wild-type males since 1-month of age and up to adult **(Fig. 2A)**. No difference was observed regarding body weights between genotypes at all ages analyzed **(Fig. 2B)**. Consistent with the

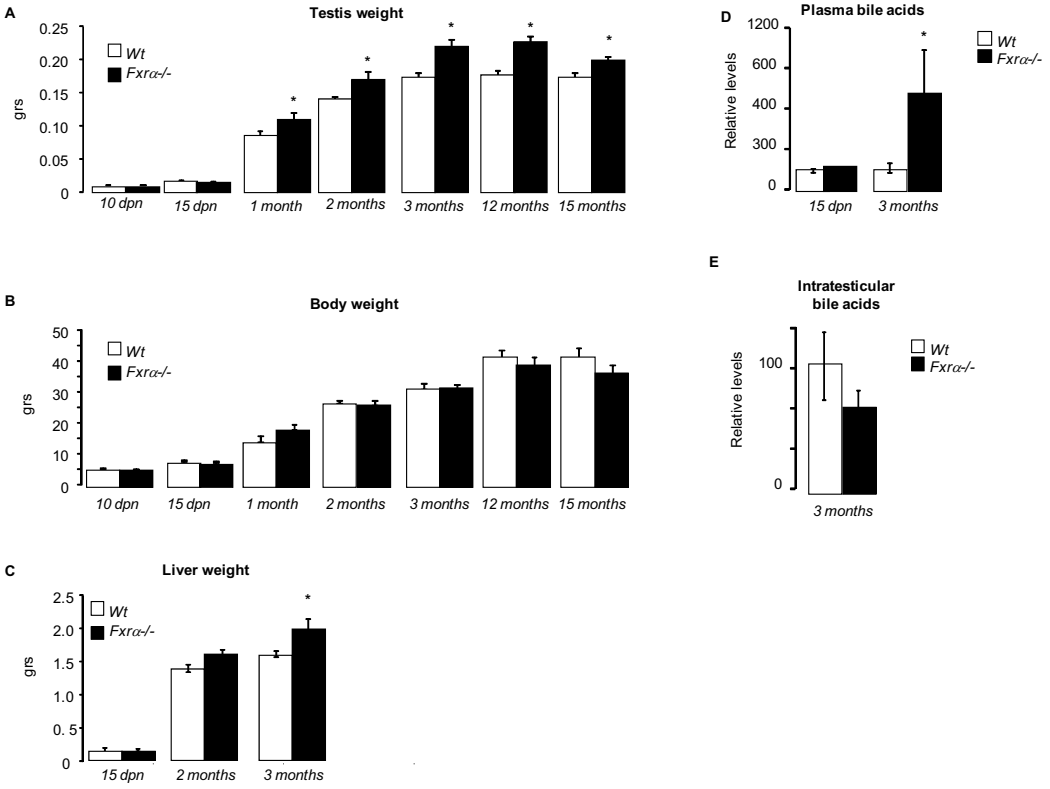

Figure 2. Lack of Fxrα alters testis relative weight. A/ Testis weight in *wild-type* and *Fxrα*$^{-/-}$ mice from 1days post-natal (dpn) to 15 months old. **B/** Body weight in *wild-type* and *Fxrα*$^{-/-}$ mice from 1-dpn to 15 months old. **C/** Liver weight in *wild-type* and *Fxrα*$^{-/-}$ mice at 15 dpn, 2 months and 15 months of age. **D/** Relative plasma bile acid levels in *wild-type* and *Fxrα*$^{-/-}$ mice at 15 dpn and 3 months of age. **E/** Relative intra-testicular bile acid levels in 3 months old wild type and *Fxrα-/-* mice. In all of the panels, n=5-10 per group; data are expressed as the means ± SEM. Statistical analysis: *, $p<0.05$.

established role of FXRα in liver physiology and BA metabolism, relative liver weight was increased in adult mice **(Fig. 2C)**. In regards to the known liver phenotype in *Fxrα*-/- mice, we decided to analyze the BA levels throughout development. *Fxrα-/-* males showed significant increase of plasma BA levels only at adult age (3 months) whereas no difference between genotype was observed at 15-dpn **(Fig. 2D)**. This is consistent with previous work published on this model showing no difference in plasma BA levels before 3 months of age in *Fxrα*-/- compared to wild-type males[22].

In addition, preliminary data did not show increased levels of BA in testis of *Fxrα*-/- males even in adult **(Fig. 2E)**. These data

suggest that the phenotypes observed at the testicular levels might not be due to an increase of testicular BA levels leading to the activation of particular BA pathways through other BA receptors such as TGR5.

Lack of FXRα alters histology of seminiferous tubules. We next studied testicular histology throughout postnatal development. Eosin-Hematoxylin approaches allow demonstrating a higher number of seminiferous tubules without open lumen in *Fxrα-/-* males compared to wild-type at 15 days postnatal (dpn) **(Fig. 3A)**.

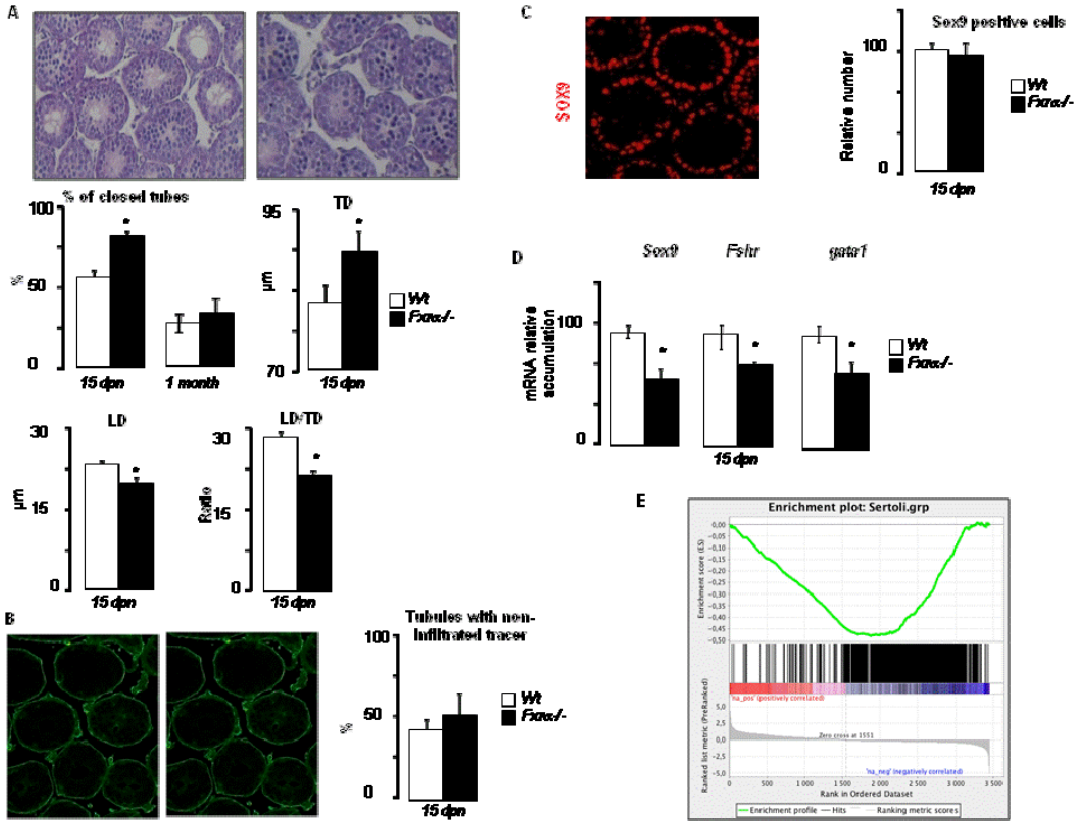

Figure 3. Lack of Fxrα alters testicular histology and Sertoli cell functions. A/ Representative micrographs of the testis of *wild-type* and *Fxrα*$^{-/-}$ mice at 15 dpn. Quantification of the percentage of tubules with closed lumen. The number of closed tubules is indicated as the number of positive cells per 100 seminiferous tubules. Evaluation of the tubule diameter (TD) and the lumen diameter (LD) and the LD/TD ratio in *wild-type* and *Fxrα*$^{-/-}$ mice at 15 dpn. **B/** Representative micrographs of the efficiency of the BTB in testis of *wild-type* and *Fxrα*$^{-/-}$ mice at 15 dpn visualized by staining of a biotynylated tracer. Quantification of the percentage of tubules with tracer infiltration. **C/** Representative micrographs of the Sox9 staining in testis of *wild-type* and *Fxrα*$^{-/-}$ mice at 15 dpn. Quantification of the number of SOX9 positive cells is indicated as the number of positive cells per 100 seminiferous tubules. **D/** Testicular mRNA accumulation of *Sox9, Fshr* and *Gata1* normalized to *β–actin* mRNA levels in the whole testes of 15 days old *wild-type* and *Fxrα*$^{-/-}$ mice. **E/** Gene selective enrichment (GSEA) in Fxrα-/- versus wild-type compared to Sertoli specific genes defined by Sanz et al[13]. In all of the panels, n=5-10 per group; data are expressed as the means ± SEM. Statistical analysis: *, $p<0.05$.

Such difference in the opening of seminiferous tubules was no more observed at 1 month of age **(Fig. 3A)**. Consistently, at 15-dpn for the tubules showing opened lumen in *Fxrα-/-* males, the diameter of the lumen was smaller compare to those of wild-type **(Fig. 3A)**. In contrast, the diameter of the seminiferous tubule was higher in *Fxrα-/-* males compare to wild-type **(Fig. 3A)**. The combination of these analyses resulted in a lower lumen diameter/tubule diameter ratio in *Fxrα-/-* males compared to wild-type animals **(Fig. 3A)**.

Lack of FXRα alters functions of Sertoli cells. In order to decipher the origin of these differences in seminiferous tubule histology between wild-type and Fxrα-/- males, we performed immunohistochemistry analyses. No difference in the efficiency of the blood-testicular barrier was observed between genotypes **(Fig. 3B)** as evaluated by immunohistochesmistry with a fluorescent tracer[14]. We studied the number of Sertoli cells which was previously demonstrated to be correlated with size of seminiferous tubules and then testis. No difference was noticed between genotypes regarding the number of Sertoli cells at 15-dpn, a time when testis alterations (opening of the lumen) were already observed **(Fig. 3C)**. In addition, the Sertoli cells have their expected localization at the periphery of the tubules **(Fig. 3C)**.

In contrast to what was observed at 15-dpn, the number of Sertoli cells per tube was reduced in adult *Fxrα*-/- males (3 months) compared to wild-type **(Suppl. 1)**. The lower number of Sertoli cells at the adult age was confirmed using another marker of Sertoli cells, e.g; GATA1 **(Suppl. 1)**. Consistently, a lower accumulation of *Sox-9* mRNA was observed in *Fxrα-/-* males compared to wild-type since 10-dpn and up to adult age **(Fig. 3D)**. In addition, the mRNA accumulation of the *Fshr* and *Occludin* were decreased in 15-dpn **(Fig.3D),** these data clearly demonstrate that the lack of *Fxrα* impacts the physiology of Sertoli cells. Rnaseq analysis highlights that a majority of genes enriched in Sertoli cells are repressed in testis of *Fxrα*-/- since 10-dpn when the number of Sertoli cells was not altered yet **(Fig. 3E)**. These data suggest the critical role of FXRα in Sertoli cell physiology.

Lack of FXRα impacts function of Leydig Cells. As Sertoli cells are dependent of androgen status and as *Fxrα* was previously described to be involved in the endocrine function of the testis at the adult age, we focused on this parameter. The males invalidated for gene encoding *Fxrα* showed lower intra-testicular and

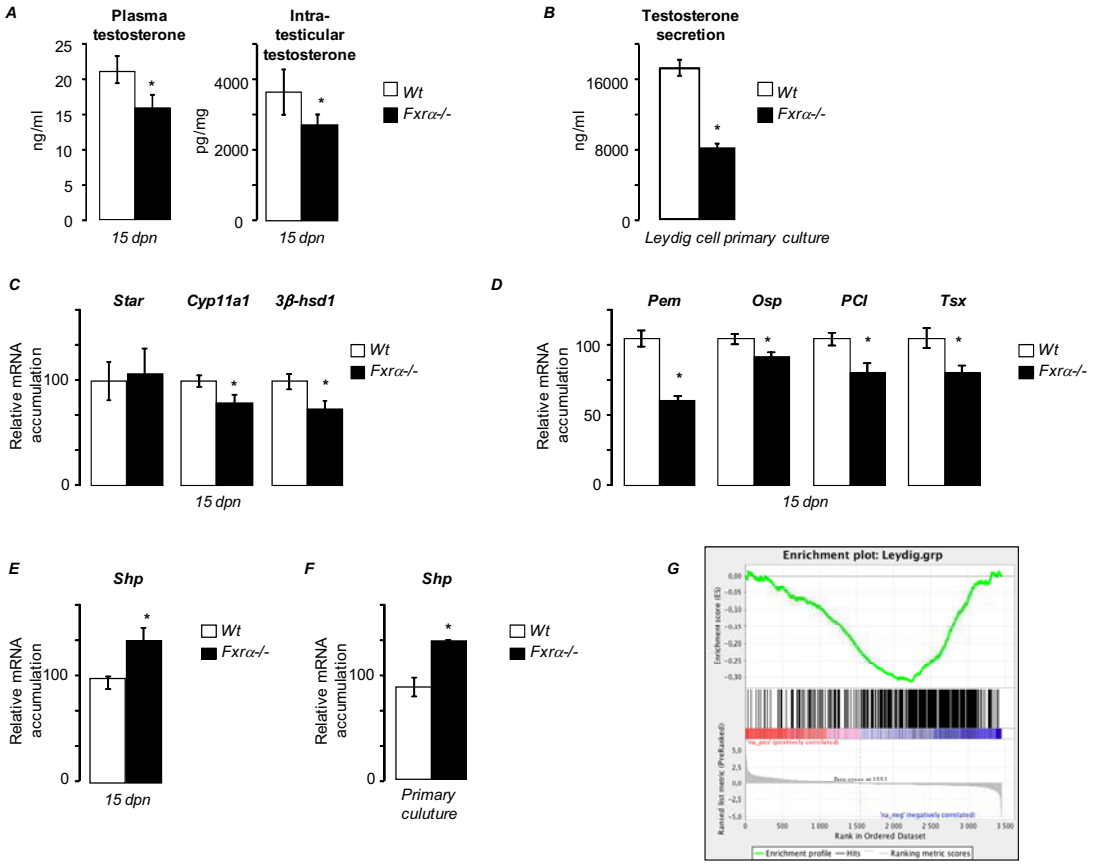

Figure 4. Leydig cell functions are deregulated in Fxrα-/- mice. A/ Plasma and intra-testicular testosterone concentrations in *wild-type* and *Fxrα*$^{-/-}$ mice at 15-dpn. **B/** Testosterone secretion in Leydig cells from adult *wild-type* and *Fxrα*$^{-/-}$ mice. **C/** Testicular mRNA accumulation of *Star, Cyp11a1* and *3b-Hsd1* normalized to *β–actin* mRNA levels in the whole testes of 15 days old *wild-type* and *Fxrα*$^{-/-}$ mice. **D/** Testicular mRNA accumulation of *Pem, Osp, Pci* and *Tsx* normalized to *β–actin* mRNA levels in the whole testes of 15 days old *wild-type* and *Fxrα*$^{-/-}$ mice. **E/** Testicular mRNA accumulation of *Shp* normalized to *β–actin* mRNA levels in the whole testes of 15 days old *wild-type* and *Fxrα*$^{-/-}$ mice. **F**/mRNA accumulation of *Shp* normalized to *β–actin* mRNA levels evaluated in Leydig cells from adult *wild-type* and *Fxrα*$^{-/-}$ mice. A/ Visualization of Gene selective enrichment (GSEA) in Fxrα-/- versus wild-type compared to Leydig specific genes defined by Sanz et al[13]. In all of the panels, n=5-10 per group; data are expressed as the means ± SEM. Statistical analysis: *, $p<0.05$.

plasma testosterone levels at 15-dpn **(Fig. 4A)**. This suggests that FXRα might control the expression of steroidogenic genes as previously demonstrated using FXRα agonist[12,article II]. In order to decipher if this effect was intrinsic to Leydig cells or involved the hypothalamo-pituitary axis, we performed primary culture of Leydig cells. A lower synthesis of testosterone by *Fxrα-/-* Leydig cells was observed compared to wild-type cultures **(Fig. 4B)**. These data on steroid levels were supported by our data showing that a lower testicular mRNA accumulation of genes involved in steroidogenesis such as *Cyp11a1* and *3β-hsd* **(Fig. 4C)**. Moreover,

the significance of such regulation was sustained by a lower mRNA accumulation of genes defined as androgen-dependent genes such as *Pem*, *Osp*, *Pci*, *Atp1a2* and *Tsx* **(Fig. 4D)**.Combined these data suggest that FXRα regulates the testicular steroidogenesis within the Leydig cells and is consistent with the observed phenotype in Sertoli cells. Such repression of steroidogenesis by *Fxrα* invalidation was surprising as activation of FXRα by synthetic agonist was shown to also repress testicular steroidogenesis [12], [article II]. These data could be reconciled as the expression of *Shp* was found increased in *Fxrα-/-* mouse **(Fig. 4E)**. Same increase in *Shp* mRNA accumulation was observed in primary culture of *Fxrα*-/- Leydig cells compared to wild-type **(Fig. 4F)**. In that line, Rnaseq analysis revealed that a majority of genes enriched in Leydig cells are repressed in testis of *Fxrα*-/- since 10-dpn **(Fig. 4G)**.

Hypotestosteronemia in *Fxrα-/-* males was not correlated with germ cell apoptosis. As testosterone levels have been demonstrated to be essential for germ cell survival, the apoptotic status of germ cell was studied. Surprisingly, a lower apoptotic rate was observed in *Fxrα-/-*mice compared to wild-type **(Fig. 5A)**.

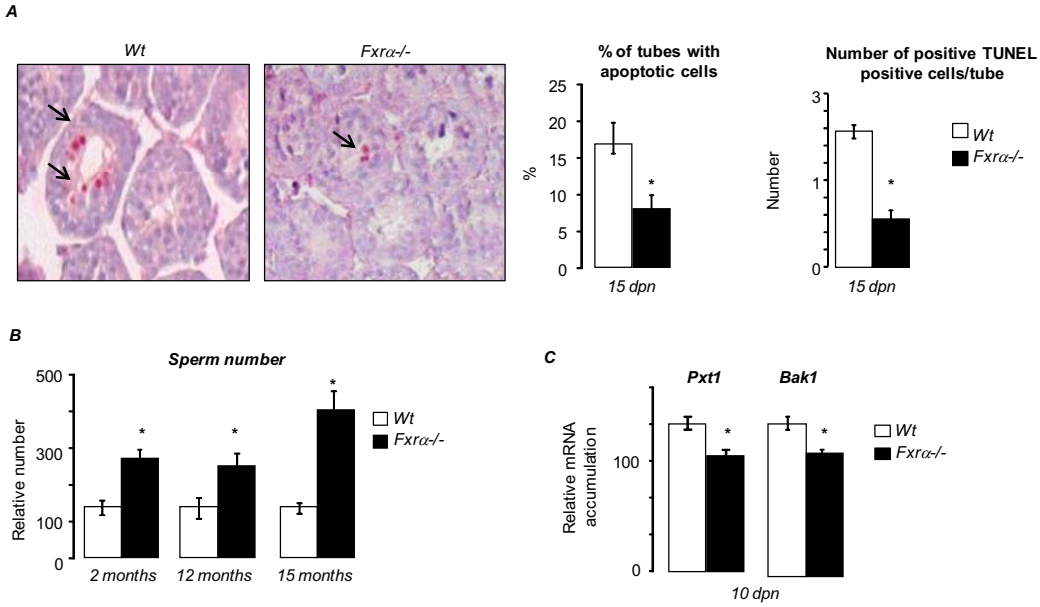

Figure 5. The lack of FXRα is associated with lower germ cell apoptosis. A/ Representative micrographs of the testis of 15-day-old *wild-type* and *Fxrα-/-* mice. The original magnification was x200. Quantification of the number of TUNEL-positive spermatocytes and TUNEL-positive tubules are indicated as the number of positive cells per 100 seminiferous tubules. **B/** Relative number of sperm count in the epididymis head in wild-type and *Fxrα-/-* at 2, 12 and 15 month-old. Wild-type were arbitrarily fixed at 100%. **C/** Testicular mRNA accumulation of *Pxt1* and *Bak1* normalized to *β–actin* mRNA levels in testes of 15 days old *wild-type* and *Fxrα-/-* mice. In all of the panels, n=5-10 per group; data are expressed as the means ± SEM. Statistical analysis: *, $p<0.05$.

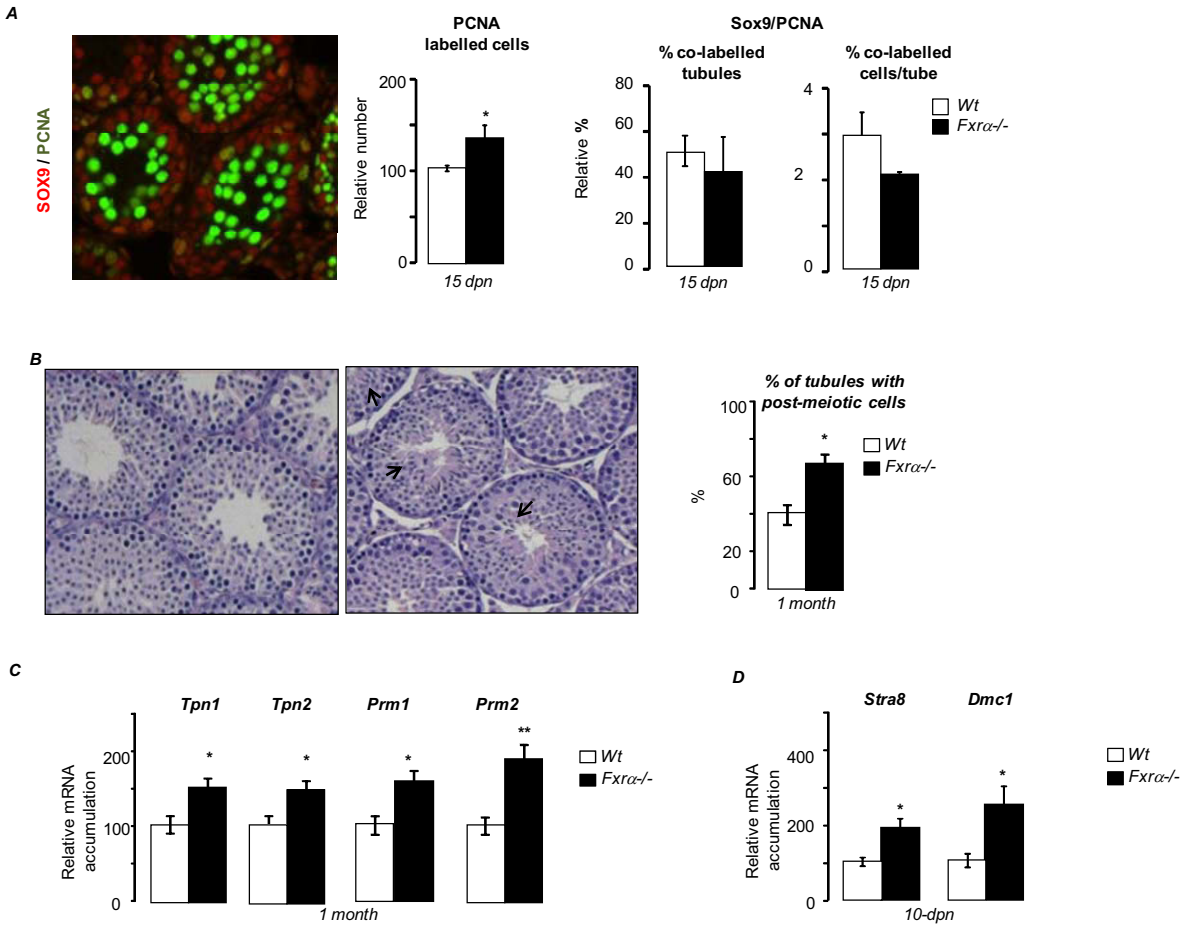

Figure 6. The lack of FXRα alters spermatogenesis process. A/ Representative micrographs of the testis of 15-day-old *wild-type* and *Fxrα-/-* mice stained for PCNA and SOX9. The original magnification was x200. Quantification of the relative number of PCNA-positive cells, the percentage of tubules co-labelled for PCNA and SOX9 as well as the percentage of cells per tubes co-labelled for PCNA and SOX9. Wild-type mice were arbitrarily fixed at 100%. **B/** Representative micrographs of the testis of 1-month-old *wild-type* and *Fxrα-/-* mice. The original magnification was x200. Quantification of the percentage of tubules showing elongated germ cells. **C/** Testicular mRNA accumulation of *Tpn1, Tpn2, Prm1* and *Prm2* normalized to *β–actin* mRNA levels in 1-month-old *wild-type* and *Fxrα-/-* mice. **D/** Testicular mRNA accumulation of *Stra8* and *Dmc1* normalized to *β–actin* mRNA levels in 1-month-old *wild-type* and *Fxrα-/-* mice. In all of the panels, n=5-10 per group; data are expressed as the means ± SEM. Statistical analysis: *, $p<0.05$.

The lower rate of germ cell apoptosis as well as the heavier testis weight was correlated with the identification of a more important production of spermatozoa by the *Fxrα-/-* mice as revealed by the counting in the head of the epididymis since 2 months of age; and this difference is still observed at 12 and 15 months old **(Fig. 5B)**. These data suggest that FXRα might participate to the control of germ cell survival through a mechanism independent of androgen status. The

Rnaseq analysis of Fxrα-/- testis allow defining several genes that have been defined as involved in germ cell apoptotic process such as *Ptx1*[23] and *Bak1*[24] **(Fig. 5C)**. The analysis of the regulation of these genes will be key in order to decipher how *Fxrα* crosstalk with germ cell apoptotic signaling. This could allow defining molecular mechanisms explaining how germ cells could survive even in the context of a hypo-testosteronemia.

FXRα-/- induces early entry into meiosis. A higher number of PCNA positive cells within the seminiferous tubules was observed 15-dpn **(Fig. 6A)**. The possibility of proliferating Sertoli cells was excluded using a co-labelling with Sox9 (Sertoli cells) and PCNA (proliferating cells) **(Fig. 6A)**. Indeed, no statistical difference was observed between genotypes in the number of proliferating Sertoli cells (**Fig. 6A)**. This suggests that the increase of PCNA positive cells was within germ cell lineage. Altogether, the presented data suggest that *Fxrα* deficiency impacts germ cell physiology. As *Fxrα*-/- produced more sperm since young age, we wonder what could be the impact of the lack of *Fxrα* on spermatogenesis. Histological analyses revealed that, at 1 month of age, a higher number of seminiferous tubules showed post-meiotic germ cells in *Fxrα*-/- compared to wild-type males **(Fig.6B).** Consistently, a higher mRNA accumulation of post-meiotic genes such as *Tpn1*, *Tpn2*, *Prm1* and *Prm2* was observed in *Fxrα-/-* males **(Fig. 6C)**. This data suggests that FXRα could participate to the control of the entry into meiosis. The first induction of genes involved in the entry into meiosis has been described to take place between 10-dpn and 15-dpn in mouse testis[12]. Consistently, testis of *Fxrα-/-* mice showed, at 10-dpn, a higher mRNA accumulation of two meiotic genes: *Stra8* and *Dmc1* **(Fig. 6D)**.

Jmjd3 is a new FXRα target gene. In line with the more important sperm production, fertility tests revealed no difference between genotypes regarding reproductive capacities as well as number of generated pups per male in mice before 12 months old **(Fig. 7A)**. However, from 12 months old, the number of wild-type fertile males was decreased **(Fig. 7A**). Indeed, a lower number of vaginal plugs (VP) generated per male was observed **(Fig. 7B)**. In contrast, old *Fxrα-/-* males showed reproductive capacities almost similar to younger mice **(Fig. 7B)**. This leads to a higher number of accumulated pups generated per males in *Fxrα*-/- genotype since 12 months old **(Fig. 7C)**.

Even if the *Fxrα* invalidation leads

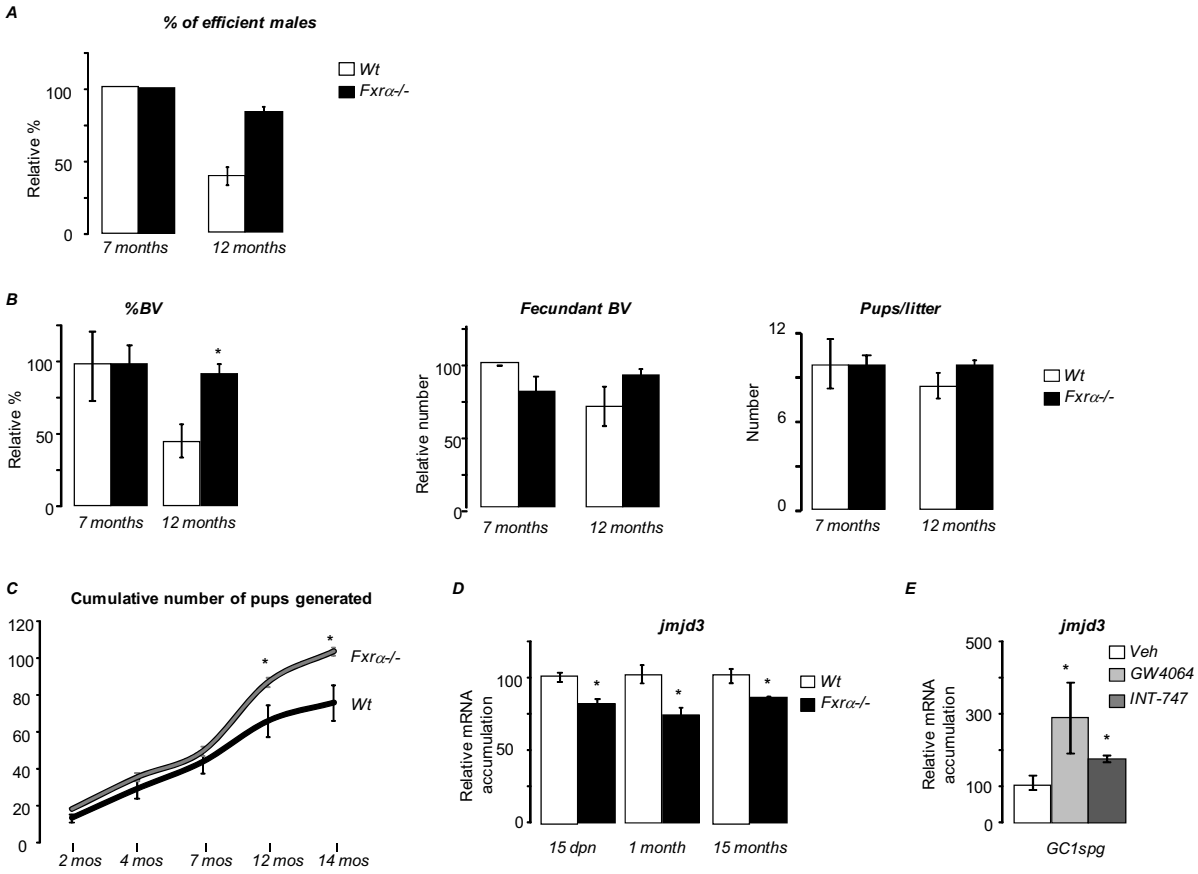

Figure 7. The lack of FXRα alters male fertility. A/ Percent of non-efficient males of 7- or 12-month-old *wild-type* and *Fxrα-/-* males after 15 days of breeding with 2 C57BL/6J females. **B/** Percentage of vaginal plugs; percentage of efficient vaginal plugs and number of pups per litter obtained for 7-month-old or 12-month-old *wild-type* and *Fxrα-/-* males. **C/** Cumulative number of pups per litter obtained for *wild-type* and *Fxrα-/-* males from 2-month-old to 14-month-old. **D/** Testicular mRNA accumulation of *Jmjd3* normalized to *β–actin* mRNA levels in 15-day-old, 1-month-old or 15-month-old *wild-type* and *Fxrα*$^{-/-}$ mice. **D/** mRNA accumulation of *Jmjd3* normalized to *β–actin* mRNA levels in GC1spg cell line treated for 8 hours with vehicle (1/1000), GW4064 (10^{-6}M) and INT747 (10^{-6}M).

In all of the panels, n=5-10 per group; data are expressed as the means ± SEM. Statistical analysis: *, $p<0.05$.

to a lower apoptotic rate of germ cells, this impact might not explain how *Fxrα-/-* could be fertile in a longer time frame compared to wild-type males. Analysis of literature leads us to focus our research on *Jmjd3*, a histone H3K27 demethylase. Indeed, it was recently demonstrated that mice invalidated for the gene encoding *Jmjd3* in germ cells show heavier testis and maintain reproductive capacities in a longer way that wild-type littermates[25]. This phenotype is closed to the one observed in *Fxrα-/-* males. Consistently, a lower mRNA accumulation of *Jmjd3* was observed in *Fxrα-/-* testis compared to wild-type at the all ages analyzed **(Fig. 7D)**. We wonder if *Jmjd3* could be an FXRα target gene. *In silico* analysis

revealed the presence of a FXRE (IR1) in the 5' regulatory sequences of *Jmjd3* gene **(data not shown)**. To define if *Jmjd3* expression could be regulated by FXRα within germ cell lineage, we used GC1-spg cell line. Treatment with FXRα agonists, GW4064 or INT747, induced a higher mRNAaccumulation of *Jmjd3* **(Fig. 7E)**.

Increased number of germ cell precursor in *Fxrα-/-* males. In order to explain how *Fxrα-/-* males could maintain higher rate of spermatogenesis compared to wild-type males, and in the line of the phenotype of the *Jmjd3-/-*, we have analyzed their potential of undifferentiated spermatogonia that are at the origin and the maintenance of germ cell lineage. Results showed a higher number of PLZF positive undifferentiated spermatogonia at 10-dpn, 15-dpn and 30-dpn **(Fig. 8A)**.

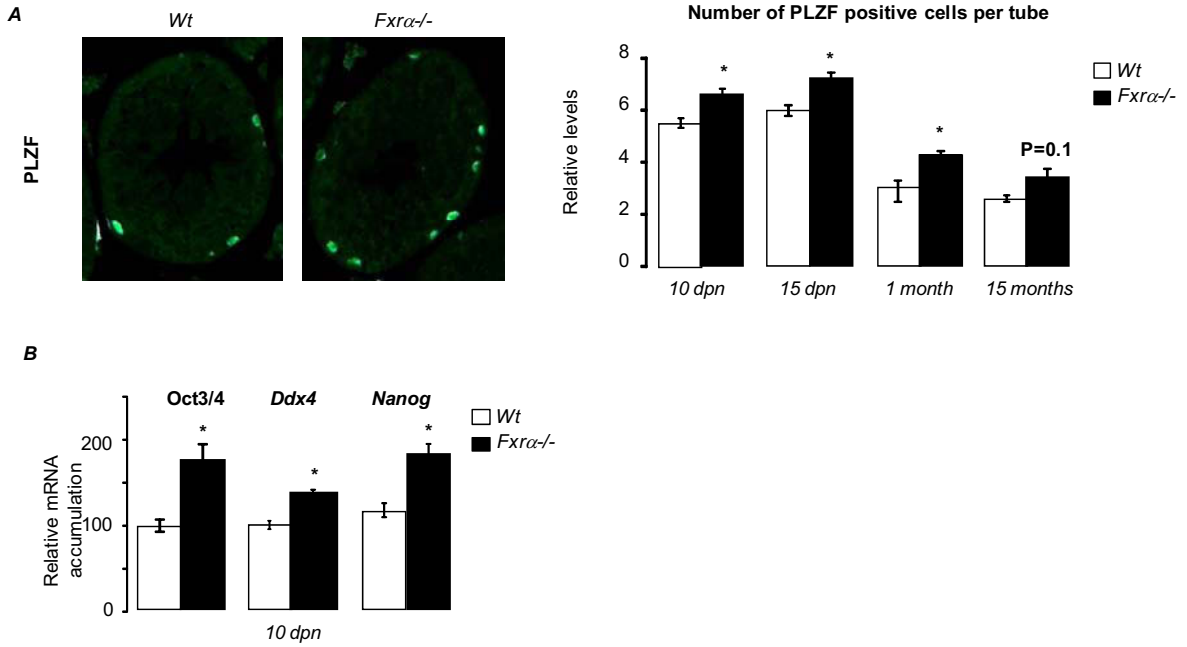

Figure 8. The lack of FXRα alters number of undifferentiated spermatogonia. A/ Representative micrographs of the testis of 15-day-old *wild-type* and *Fxrα-/-* mice stained for PLZF. The original magnification was x200. Quantification of the relative number of PLZF-positive cells per tubes at 10-dpn, 15-dpn, 1-month-old or 15-month-old. **B/**Testicular mRNA accumulation of *Oct3/4, Ddx4* and *Nanog* normalized to *β–actin* mRNA levels in 10-day-old *wild-type* and *Fxrα-/-* mice.

In all of the panels, n=5-10 per group; data are expressed as the means ± SEM. Statistical analysis: *, $p<0.05$.

This tendency was confirmed at 15 months old **(Fig. 8A)**. These data suggest that *Fxrα* deficiency might participate to spermatogonial stem cell fate. The analysis of a RNAseq experiment of 10-dpn testis revealed alteration of mRNA accumulation of genes present in undifferentiated germ cells such as *Oct3/4*, *Nanog*, *Dazl* or *Vasa*

(Fig. 8B). In addition, several *Hox* genes were also found upregulated in *Fxrα-/-* testis compared to wild-type mice **(data not shown)**. These data support the idea of an increase of the undifferentiated germ cell population in the context of the lack of FXRα.

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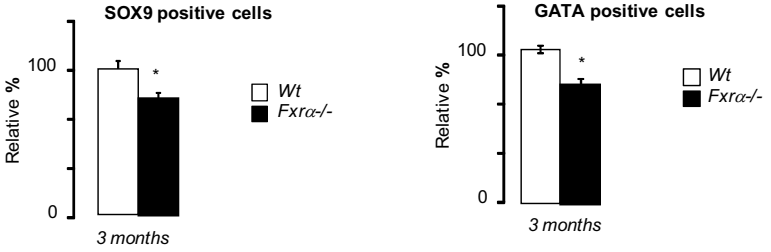

Supplemental. A/ Quantification of the relative number of SOX9 or GATA1-positive cells per tube at 3-month-old *wild-type* and *Fxrα-/-* mice.

In all of the panels, n=5-10 per group; data are expressed as the means ± SEM. Statistical analysis: *, $p<0.05$.

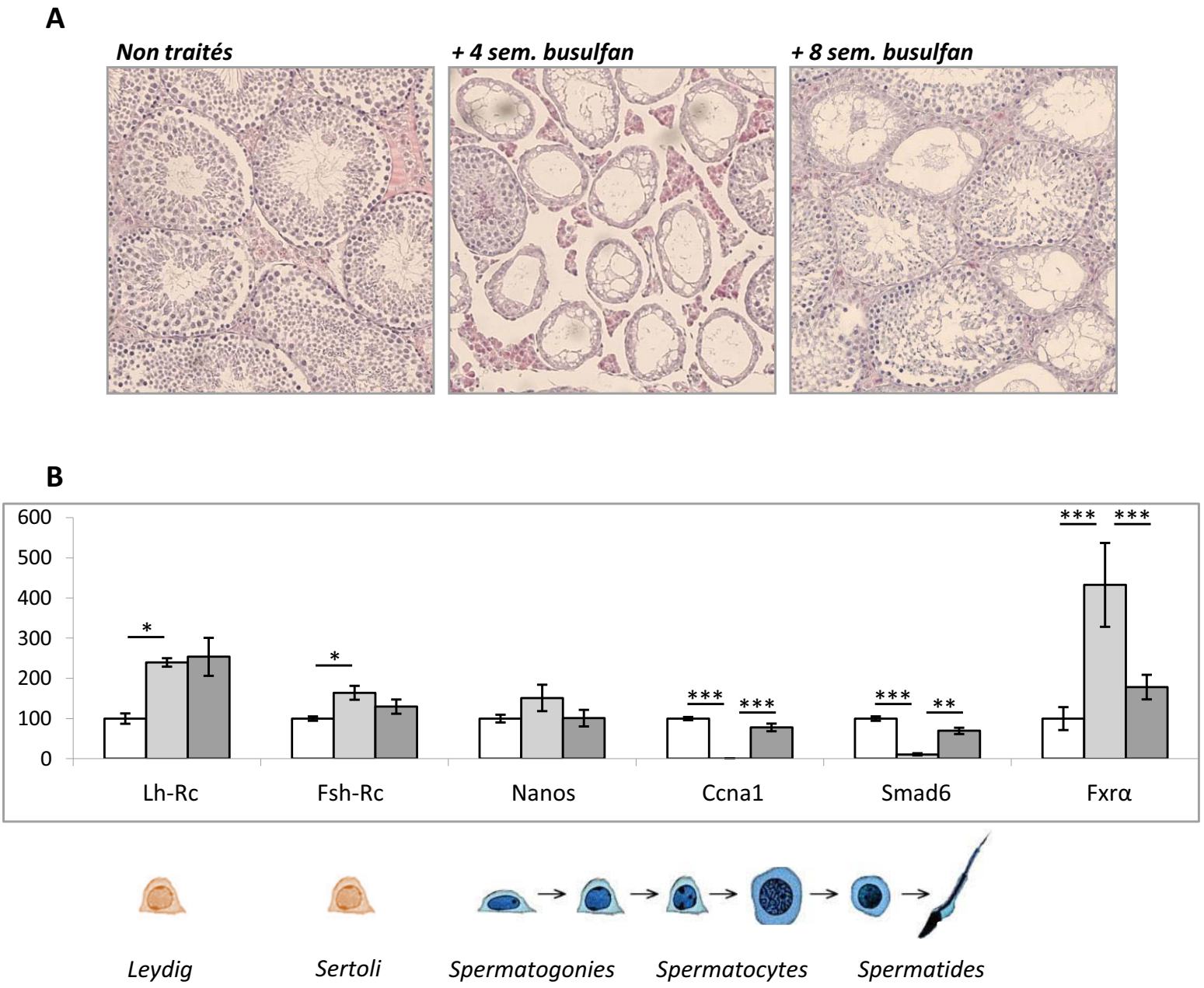

Figure 19 : Analyse de l'impact du busulfan sur l'histologie testiculaire et l'expression de marqueurs spécifiques aux différents types cellulaires du testicule chez la souris mâle adulte. A. Analyse histologique. Le busulfan entraîne, 4 semaines après son injection, une perte transitoire de la lignée germinale, à l'exception des spermatogonies qui permettront la recolonisation quasi-totale des tubes séminifères 8 semaines après le début du traitement. **B.** Analyse moléculaire. Ces altérations histologiques s'accompagnent au niveau moléculaire d'un effondrement de l'accumulation des transcrits de marqueurs spécifiques des cellules méiotiques (Ccna1) et post-méiotiques (Smad6), tandis que celle de marqueurs des cellules somatiques (Lh-Rc et Fsh-Rc) et des spermatogonies (Nanos) augmente en proportion 4 semaines après l'injection du busulfan. Après 4 semaines supplémentaires (8 semaines post-injection), le profil d'expression de l'ensemble de ces marqueurs montre un retour à la normale en conséquence de la recolonisation du testicule par la lignée germinale. L'accumulation des transcrits du gène codant Fxrα augmente, quant-à elle, 4 semaines après le début du traitement, de façon beaucoup plus marquée par rapport aux marqueurs Lh-Rc, Fsh-Rc et Nanos.
Lh-Rc : Luteinizing Hormone-Receptor ; Fsh-Rc : Follicle Stimulating Hormone-Receptor ; Ccna1 : Cyclin A1 ; Fxrα : Farnesoid X Receptor alpha.

Discussion et perspectives de l'article III

Nous montrons dans ce troisième article que les deux fonctions majeures du testicule, exocrine et endocrine, sont altérées chez les animaux Fxrα$^{-/-}$. Fxrα apparaît par ailleurs comme un nouvel acteur impliqué dans le contrôle du nombre et du caractère pluripotent des cellules germinales souches.

1. Où est Fxrα dans le testicule ?

Deux études indépendantes ont par le passé identifié Fxrα dans les cellules de Leydig chez la souris adulte (33)-(122). Nous montrons que Fxrα est exprimé dans le testicule dès la naissance, et jusqu'à l'âge adulte. Cependant, l'étude de sa localisation précise dans cet organe est rendu difficile par le manque d'anticorps spécifique dirigé contre ce récepteur. Nous avons donc opté pour des méthodes alternatives afin de tenter de préciser le profil d'expression de Fxrα dans le testicule.

a) L'ontogénie

Le profil d'expression de Fxrα dans le testicule murin tout au long de la vie, présenté dans ce troisième article, ne se superpose avec aucun de celui des différents marqueurs spécifiques analysés : Lh-Rc pour les cellules de Leydig, Fsh-Rc pour les cellules de Sertoli, et G9a pour les spermatogonies. Il semble donc que Fxrα soit exprimé dans plusieurs types cellulaires du testicule, et que sa localisation varie au cours du temps. Le profil d'expression de Fxrα est en effet similaire à celui de G9a au cours du développement post-natal précoce, et semblable à celui du Lh-Rc et Fsh-Rc à partir de 25 jours post-nataux. A partir de cet âge, l'expression de Fxrα diminue, ce qui va à l'inverse de celle de marqueurs de cellules méiotiques et post-méiotiques (données non montrées) qui augmente de façon considérable du fait du démarrage de la spermatogenèse.

b) Utilisation du busulfan.

Le busulfan est une molécule reprotoxique utilisée pour le traitement de la leucémie myéloïde chronique. Il entraîne chez la souris mâle adulte, 4 semaines après son injection, une perte transitoire de la lignée germinale, à l'exception des spermatogonies qui permettront la recolonisation quasi-totale des tubes séminifères 8 semaines après le début du traitement à la dose utilisée (**Figure 19**). Ces altérations histologiques s'accompagnent au niveau moléculaire d'un effondrement de l'accumulation des transcrits de marqueurs spécifiques des cellules méiotiques (Ccna1) et post-méiotiques (Smad6), tandis que celle de marqueurs des cellules somatiques (Lh-Rc et Fsh-Rc) et des spermatogonies (Nanos) augmente en proportion 4 semaines après l'injection du busulfan. Après 4 semaines supplémentaires (8 semaines post-injection), le profil d'expression de l'ensemble de ces marqueurs montre un retour à la normale en conséquence de la recolonisation du testicule par la

lignée germinale. L'accumulation des transcrits du gène codant Fxrα augmente, quant-à elle, 4 semaines après le début du traitement, de façon beaucoup plus marquée par rapport aux marqueurs Lh-Rc, Fsh-Rc et Nanos, suggérant à nouveau qu'il puisse être exprimé dans plusieurs des types cellulaires correspondant (Leydig, Sertoli, Spermatogonies). Il semble par ailleurs peu probable au vu de ces résultats que Fxrα soit exprimé dans les cellules méiotiques (spermatocytes) et post-méiotiques (spermatides).

c) Fxrα dans la lignée germinale

Grâce à une collaboration avec l'équipe du Pr. Kochbin (Grenoble, France), nous avons pu avoir accès à des échantillons murins de tri cellulaire correspondant à différents stades de la lignée germinale (cellules germinales totales, spermatocytes, spermatides rondes et cellules allongées). Nous avons montré que Fxrα est enrichi dans un échantillon de testicule total par rapport à ces différents échantillons germinaux dans lesquels il est peu ou pas détecté. Ces résultats n'excluent cependant pas son expression dans les spermatogonies (échantillon auquel nous n'avons pu avoir accès), mais sont en revanche en accord avec l'analyse de l'ontogénie et du busulfan qui rend peu probable la possibilité qu'il soit exprimé dans les stades plus tardifs de la spermatogenèse (spermatocytes, spermatides).

c) Le tri cellulaire

L'ensemble de ces analyses localiseraient le récepteur nucléaire Fxrα dans les cellules de Leydig (conformément à de précédentes études), et potentiellement dans les cellules de Sertoli et les spermatogonies. Elles ne sont cependant pas satisfaisantes. *Nous envisageons de réaliser un tri magnétique de différents types cellulaires, et espérons pouvoir trancher définitivement quant-au profil d'expression de Fxrα dans le testicule à l'aide de ce support technique*. Ces résultats seront primordiaux quant-à la compréhension des mécanismes moléculaires impliqués dans les altérations testiculaires observées chez les animaux Fxrα$^{-/-}$. *Ceux-ci pourront être appréhendés in vitro à l'aide des lignées cellulaires murines MA10, 42GPA9 et GC1-spg correspondant respectivement aux cellules de Leydig, de Sertoli et germinales (spermatogonies/spermatocytes), dans chacune desquelles nous avons détecté la présence de Fxrα.*

2. Invalidation de Fxrα et altérations testiculaires : un lien local ou systémique ?

Les animaux Fxrα$^{-/-}$ présentent des désordres métaboliques importants à l'âge adulte, se traduisant par une dérégulation de l'homéostasie des acides biliaires, une élévation des concentrations de cholestérol et de triglycérides (71), ainsi qu'une intolérance au glucose couplée à une résistance à l'insuline (72)-(32)-(73). Nous montrons qu'à 10-15 jours post-nataux, âge auquel la physiologie

testiculaire est déjà altérée, les concentrations plasmatiques d'acides biliaires sont équivalentes chez les souris sauvages et Fxrα$^{-/-}$. Ces résultats sont en accord avec les travaux de Anakk *et al.* (2011) qui ont montré que l'élévation plasmatique de la concentration d'acides biliaires n'est observable chez les animaux Fxrα$^{-/-}$ qu'à partir de l'âge de 12 semaines (402). De plus, des données préliminaires montrent qu'aucune variation de la quantité d'acides biliaires intra-testiculaire n'est observable chez les animaux Fxrα$^{-/-}$. Cela exclut une augmentation de la signalisation des acides biliaires *via* un autre de leurs récepteurs, ou d'un effet toxique de ces molécules de part leur action détergente, comme étant à l'origine du phénotype observé.

Qu'en est-il des autres perturbations systémiques observées chez les animaux Fxrα$^{-/-}$? Sont-elles détectables dès l'âge de 10-15 jours, et participent-elles à l'altération de la physiologie testiculaire observée à cet âge ? En effet, celle-ci se traduit principalement par la répression de la stéroïdogenèse et l'altération du nombre des cellules germinales souches ; or, une modification des concentrations de cholestérol, le substrat de la stéroïdogenèse, ainsi que des triglycérides (cf paragraphe **3.b)** de cette discussion) pourrait notamment altérer la synthèse des androgènes. *Le dosage plasmatique de ces métabolites devrait donc être fait.*

Plusieurs arguments viennent cependant appuyer l'hypothèse selon laquelle l'absence de Fxrα elle-même dans le testicule pourrait être responsable des altérations testiculaires mises en évidence chez les animaux Fxrα$^{-/-}$.

a) Fxrα et stéroïdogenèse

Nous montrons, de façon cohérente avec la littérature, que Fxrα est impliqué dans le contrôle de la stéroïdogenèse. En effet, nous mettons en évidence une diminution des concentrations intra-testiculaire et plasmatique de testostérone chez les animaux Fxrα$^{-/-}$ par rapport aux animaux sauvages. Il est intéressant de noter que l'activation (Volle *et al*. (2007), article II) et la perte (article III) de Fxrα aboutissent toutes deux à la répression de la stéroïdogenèse : cet aspect inattendu sera développé dans la discussion générale de ce manuscrit. Au niveau moléculaire, cette altération est corrélée à une dérégulation de l'expression des gènes codant les enzymes stéroïdogènes (Cyp11a1, 3β-Hsd) et des gènes dits androgéno-dépendants (Pem, Osp, Pci, Tsx, Atp1a2). Cet impact de Fxrα sur l'activité endocrine des cellules de Leydig semble être intrinsèque à ce type cellulaire, comme l'atteste la diminution de la synthèse et de la sécrétion de testostérone par des cellules de Leydig primaires issues de souris Fxrα$^{-/-}$ adultes par rapport à celles issues de mâles sauvages. *Le dosage plasmatique des gonadotrophines (Lh et Fsh) devrait cependant être réalisé chez les animaux sauvages et Fxrα$^{-/-}$ de 15 jours post-nataux afin de déterminer si une dérégulation de l'axe central*

hypothalamo-hypophysaire pourrait participer malgré tout à la répression de la stéroïdogenèse observée chez les animaux Fxrα$^{-/-}$.

b) Fxrα et cellules germinales souches

Une augmentation du nombre de cellules germinales souches est observée chez les animaux Fxrα$^{-/-}$ par rapport aux animaux sauvages. A notre connaissance, aucune des altérations métaboliques systémiques observées chez les animaux Fxrα$^{-/-}$ n'est associée à une variation du nombre de ces cellules. Par ailleurs, l'exposition de souris mâles sauvages au cours du développement fœtal et post-natal à un antagoniste de fxrα, le stigmastérol, reproduit ce phénotype (données non publiées). Ces résultats suggèrent que l'altération de la signalisation associée à fxrα, par perte d'expression (modèle KO) ou d'activité (stigmastérol), influence elle-même le nombre de cellules germinales indifférenciées. En revanche, comme développé précédemment, si ce paramètre est induit par une diminution de la signalisation associée à Fxrα au sein des cellules germinales souches et / ou d'un autre type cellulaire du testicule, reste encore à être déterminé.

L'invalidation du gène codant Fxrα spécifiquement dans un ou plusieurs types cellulaires du testicule permettrait d'écarter ou non de façon définitive l'implication de facteurs systémiques dans les altérations testiculaires observées chez les animaux Fxrα$^{-/-}$. Cette alternative pourra être envisagée lorsque la localisation de Fxrα dans le testicule aura été précisée. Le croisement de souris dont le gène codant Fxrα est flanqué de séquences Lox, avec des souris exprimant la recombinase Cre sous la dépendance du promoteur du gène codant le récepteur à l'Amh (Amhr2-Cre), l'Amh (Amh-Cre) ou la protéine de liaison aux ARN Vasa (Vasa-Cre) nous permettra alors d'invalider le gène codant Fxrα spécifiquement dans les cellules de Leydig, de Sertoli ou dans les spermatogonies souches respectivement.

3. Par quels mécanismes moléculaires Fxrα réprime-t-il la stéroïdogenèse ?

a) Implication de la voie Shp-Dax-1 / Lrh-1-Sf-1 ?

Les travaux de Volle *et al*. (2007), ainsi que les résultats de l'article II montrent que Fxrα contrôle la stéroïdogenèse par le biais de deux voies moléculaires probablement distinctes :

-Fxrα réprime l'expression des enzymes stéroïdogènes en inhibant l'activité transcriptionnelle des récepteurs Lrh-1 et Sf-1 par le biais de Shp et / ou Dax-1 ;

-Fxrα altère la synthèse des stéroïdes en diminuant la sensibilité des cellules de Leydig à la Lh.

De tels mécanismes sont-ils mis en jeu dans la répression de la stéroïdogenèse observée chez les animaux fxrα$^{-/-}$? *Cette question devrait être approchée par l'analyse de l'expression des gènes codant : Shp, Dax-1, Lrh-1 et Sf-1 (hypothèse 1), le récepteur à la Lh (hypothèse 2).* Notons que l'expression de Shp est augmentée de façon significative *in vivo* (testicule total d'animaux de 15 jours) et *ex vivo* (culture primaire de cellules de Leydig) dans les échantillons issus de souris fxrα$^{-/-}$ par rapport à ceux issus d'animaux sauvages. Par ailleurs, *l'analyse de la réponse des gènes codant les enzymes stéroïdogènes à une injection d'hCG chez les animaux sauvages et fxrα$^{-/-}$ devraient permettre de déterminer si la sensibilité des cellules de Leydig est altérée du fait de la perte de Fxrα (hypothèse 2).*

b) Et les triglycérides dans tout ça ?

Les travaux de Watanabe *et al*. (2004) ont montré que Fxrα est impliqué dans le contrôle de la production hépatique des triglycérides. En effet, il conduit à l'activation de Shp, qui en inhibant l'activité transcriptionnelle des récepteurs nucléaires Lxr, réprime l'expression du facteur de transcription Srebp-1c et de ses gènes cibles (Fas, AceCS et Scd1) impliqués dans la synthèse des acides gras et des triglycérides (31). L'ensemble de ces acteurs sont exprimés dans le testicule, et en particulier dans les cellules de Leydig. La synthèse des acides gras et des triglycérides est-elle soumise à une telle régulation par Fxrα dans ce type cellulaire ? Ce questionnement trouve tout son sens au vu des travaux de Meikle *et al*. (1989) (403), ayant montré que les triglycérides et acides gras non estérifiés (acides linoléique, stéarique, oléique et palmitique) inhibe la synthèse de testostérone en réponse à la Lh dans une culture primaire de cellules de Leydig murines. Ainsi, une augmentation de la synthèse testiculaire de ces composés lipidiques en réponse à l'absence de Fxrα, pourrait-elle participer à la répression de la stéroïdogenèse observée chez les animaux Fxrα$^{-/-}$. *L'analyse de l'expression des gènes Shp, Srebp-1c, Fas, AceCS, Scd1 dans les cellules de Leydig primaire issues de souris sauvages et Fxrα$^{-/-}$, ainsi que le dosage des triglycérides dans ces mêmes cellules devraient permettre de vérifier cette hypothèse.* Nos résultats préliminaires montrent dans ces échantillons cellulaires une augmentation de l'expression de Shp mais associée à une diminution de l'accumulation des transcrits des gènes codant Acc et Fas. La synthèse des triglycérides dans les cellules de Leydig semble donc être diminuée chez les animaux Fxrα$^{-/-}$ par rapport aux animaux sauvages, ce qui exclurait leur implication dans la répression de la stéroïdogenèse.

4. Fxrα et apoptose germinale

Nous mettons en évidence une hypo-androgénie intra-testiculaire chez les animaux Fxrα$^{-/-}$ par rapport aux animaux sauvages. Une telle altération s'accompagne normalement de l'induction d'une apoptose massive des cellules germinales, en particulier des stades méiotiques. De façon

surprenante, les animaux Fxrα$^{-/-}$ présentent non pas une augmentation, mais une nette diminution de l'apoptose germinale par rapport aux animaux sauvages, et ce dès l'âge de 15 jours et jusqu'à l'âge adulte (15 mois). Fxrα contrôle-t-il l'expression d'un ou plusieurs acteur(s) impliqué(s) dans le processus apoptotique de la lignée germinale ? L'analyse du *RNA Seq* a révélé la dérégulation de l'expression des facteurs pro-apoptotiques Bak1 et *Peroxisomal testis-specific 1* (Pxt1) (404)-(405). Des expérimentations *in vitro* sont envisagées afin de préciser le rôle potentiel joué par la diminution de l'expression de ces acteurs :

1- des cellules GC1-spg (spermatogonies / spermatocytes) seront traitées avec un agoniste de Fxrα (GW4604, INT-747, CDCA) : l'apoptose des cellules en réponse à l'activation de Fxrα sera alors évaluée grâce à un marquage TUNEL, et l'expression de Bak1 et PXT1 par RT-qPCR ;

2- dans l'hypothèse où un tel traitement induirait une apoptose des cellules et une augmentation de l'expression de l'un et / ou l'autre de ces gènes, il sera reproduit en présence d'un siRNA dirigé contre :

-Fxrα pour s'assurer que la potentielle action pro-apoptotique des agonistes utilisés est bien le résultat de l'activation de Fxrα ;

-Bak1 et / ou PXT1 pour s'assurer que la potentielle action pro-apoptotique des agonistes utilisés résulte bien de l'activation de l'un et / ou l'autre de ces gènes pro-apoptotiques.

Par ailleurs, nous avons identifié un élément de réponse à Fxrα (de type IR1) dans le promoteur du gène codant Bak1 grâce à l'utilisation du logiciel Genomatix. Afin de déterminer si Bak1 est un nouveau gène cible de Fxrα, plusieurs techniques pourront être employées dans les cellules GC1-spg :

-une immunoprécipitation de la chromatine sera réalisé afin de déterminer si Fxrα est capable de se fixer sur le promoteur du gène codant Bak1 ;

-le promoteur du gène codant Bak1 sera cloné en amont du gène codant la luciférase, dont l'activité sera mesurée en réponse à l'ajout d'un agoniste de Fxrα.

5. Fxrα et cellules germinales souches

Nous mettons en évidence chez les animaux Fxrα$^{-/-}$ une augmentation du nombre de cellules germinales indifférenciées, positives pour le marqueur Plzf, par rapport aux animaux sauvages. Un tel phénotype a été observé par Iwamori *et al*. (2013) chez un modèle murin dont le gène codant l'histone déméthylase Jmjd3 a été invalidé spécifiquement dans la lignée germinale (406). Ils en ont

identifié l'origine, grâce à des approches *in vitro* et *in vivo*, mettant en évidence un processus de dé-différenciation des spermatogonies différenciées à la suite d'une fragmentation des cystes germinaux, par des mécanismes moléculaires qu'il reste à identifier. En effet, trois équipes indépendantes avaient démontré auparavant chez la souris que les spermatogonies différenciées étaient capables de ''revenir en arrière'' quant-au processus de différenciation à la suite d'une rupture des ponts inter-cellulaires les reliant les unes aux autres, permettant de cette façon un renouvellement rapide et stochastique du pool de cellules germinales souches (407)-(408)-(409).

De façon intéressante, nous avons mis en évidence une diminution de l'accumulation des transcrits du gène codant Jmjd3 dans le testicule de souris Fxrα$^{-/-}$ à différents âges par rapport aux animaux sauvages, pouvant expliquer l'origine de l'augmentation du nombre de cellules indifférenciées. *L'analyse de l'intégrité des ponts intercellulaires par immunohistochimie sur les tubes séminifères entiers devraient être réalisée afin de s'assurer de l'implication de ce processus dans notre phénotype.* Plusieurs questions se posent alors : par quels mécanismes moléculaires l'expression de Jmjd3 est-elle altérée ? D'autres processus participent-ils à l'augmentation du nombre de cellules germinales souches ?

a) Jmjd3 : un nouveau gène cible de Fxrα ?

Afin de comprendre les mécanismes moléculaires mis en jeu dans l'altération de l'expression du gène codant Jmjd3 observée chez les souris Fxrα$^{-/-}$, nous avons traité pendant 8 heures une lignée cellulaire spermatogoniale murine (GC1-spg) avec deux agonistes synthétiques de Fxrα, le GW4064 et l'INT-747. Les données préliminaires montrent une augmentation de l'expression de Jmjd3 en réponse à l'un ou l'autre de ces agonistes. De plus, nous avons identifié un élément de réponse à Fxrα (IR1) dans le promoteur du gène codant Jmjd3. L'ensemble des ces analyses pourraient définir Jmjd3 comme un nouveau gène cible du récepteur nucléaire Fxrα, *et cette hypothèse devrait être vérifiée par une approche méthodologique identique à celle envisagée pour Bak1.*

b) Une altération des capacités d'auto-renouvellement et / ou de différenciation des spermatogonies participe-t-elle à l'augmentation du pool de cellules germinales indifférenciées chez les animaux Fxrα$^{-/-}$?

L'implication de la diminution de l'expression de Jmjd3 et de la rupture des cystes germinaux pour expliquer l'augmentation du nombre de cellules germinales indifférenciées n'exclue pas un impact de la perte de Fxrα sur les capacités d'auto-renouvellement et / ou de différenciation de ces cellules.

L'augmentation du nombre de cellules souches pourrait résulter d'une augmentation de leur auto-prolifération et / ou d'un blocage de leur différenciation, ce qui conduirait à une diminution du

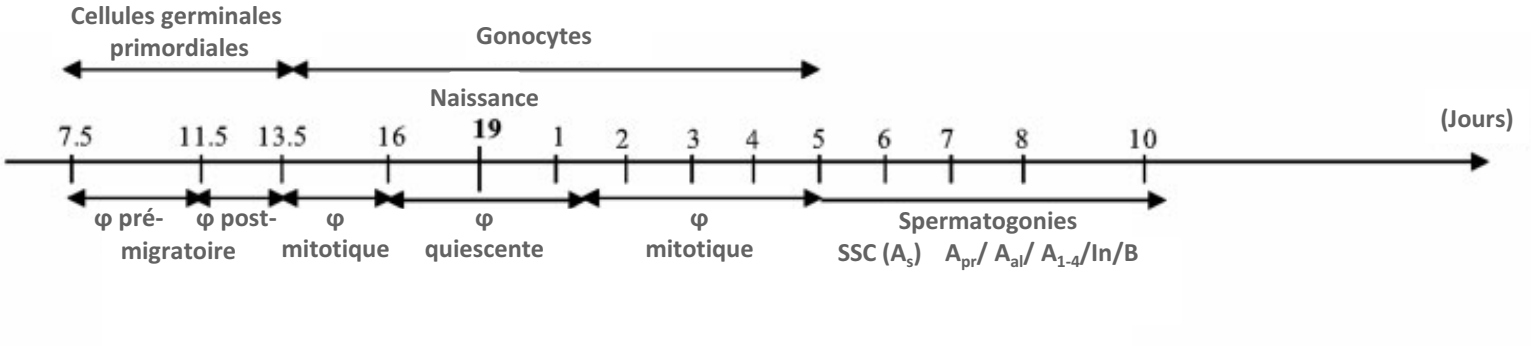

Figure 20 : Représentation schématique du développement des gonocytes. Les gonocytes dérivent des cellules germinales primordiales. Ils subiront une phase de prolifération au cours du développement fœtal puis néo-natal avant de se différencier en cellules germinales souches (SSC).
φ *: Phase ; SSC : Spermatogonial Stem Cell : Spermatogonies* A_s *:* A_{single} *;* A_{pr} *:* A_{paired} *;* A_{al} *:* $A_{aligned}$ *; In : Intermédiaire.* Adapté d'après Culty et al. 2009.

nombre de cellules s'engageant dans la voie de la spermatogenèse. Au contraire, les mâles Fxrα$^{-/-}$ produisent un nombre plus important de spermatozoïdes que les animaux sauvages, ce qui invaliderait cette hypothèse.

c) La perte de Fxrα favorise-t-elle la prolifération des gonocytes ?

Les cellules souches germinales dérivent des gonocytes au cours de la première semaine post-natale, eux-mêmes issus de la différenciation des cellules primordiales germinales (**Figure 20**). Une augmentation de la prolifération fœtale des cellules primordiales germinales et / ou de la prolifération fœtale et néo-natale des gonocytes pourrait donc être responsable de l'augmentation du nombre de cellules germinales souches observée chez les animaux Fxrα$^{-/-}$. Cette hypothèse est soutenue par le fait que Fxrα a été détecté dans le testicule fœtal humain (410). *Nous utiliserons un marqueur de cellules pluripotentes (Oct3/4) afin de réaliser un comptage histologique de ces cellules au cours du développement embryonnaire et de la période néo-natale.*

Lorsque nous aurons déterminé à partir de quelle étape développementale (fœtale, néo-natale, post-natale) le nombre de précurseurs des cellules germinales souches, ou des cellules germinales souches elles-mêmes est augmenté, nous pourrons préciser les mécanismes moléculaires impliqués.

6. Fxrα et cellules de Sertoli

Nous montrons que la perte de Fxrα altère à la fois le nombre et les fonctions des cellules de Sertoli.

a) Altération du nombre de cellules de Sertoli

Une diminution du nombre des cellules de Sertoli est observée à l'âge adulte, alors que celle-ci n'est pas encore altérée chez les animaux de 15 jours, âge auquel ces cellules continuent de proliférer. Ce phénotype pourrait s'expliquer par un arrêt prématuré de la prolifération des cellules de Sertoli au cours du développement pubertaire.

1-Le taux de prolifération de ces cellules sera donc évalué chez des animaux âgés de 20 jours à l'aide d'un co-marquage PCNA / SOX9 (PCNA étant un marqueur de prolifération, et SOX9 un marqueur exprimé spécifiquement dans les cellules de Sertoli).

2-L'hormone thyroïdienne T3 serait un acteur prépondérant responsable de l'arrêt de la prolifération des cellules de Sertoli. *Une intégration potentiellement prématurée du signal de cette hormone sera déterminée par l'évolution de sa concentration plasmatique au cours du dévelopement post-natal, ainsi que par l'analyse du profil d'expression de son récepteur TRα, et de l'acteur p27^{kip1} impliqué dans le déclenchement de l'arrêt du cyle cellulaire des cellules de Sertoli.*

3-L'acide rétinoïque réprime *in vitro* la prolifération des cellules de Sertoli et favorise le processus de maturation. *L'implication potentielle des rétinoides dans l'arrêt prématuré supposé de la prolifération des cellules de Sertoli sera évaluée par un dosage intra-testiculaire, et par l'analyse de l'expression de leur récepteurs et gènes cibles.* Une telle augmentation de la signalisation des rétinoïdes pourrait par ailleurs expliquer, au moins en partie, le démarrage précoce de la spermatogenèse observée chez les animaux fxr$\alpha^{-/-}$.

b) Altération des fonctions Sertoliennes

Nous montrons en plus de leur nombre, une altération de la fonction des cellules de Sertoli comme l'atteste la diminution de l'expression de nombreux gènes enrichis dans les cellules de Sertoli (analyse GSEA). Cela se traduit notamment à 15 jours par un retard d'ouverture de la lumière des tubes, processus dépendant en partie des androgènes intra-testiculaires. Ainsi, l'hypo-androgénie observée chez les animaux fxrα-/- pourrait-elle participer à ce retard de canalisation des tubes séminifères ? *Une injection de testostérone chez ces animaux de la naissance à 15 jours devrait permettre de valider ou non cette hypothèse.*

DISCUSSION ET PERSPECTIVES GENERALES

Même si des perspectives immédiates ont été proposées dans la discussion de chaque article présenté, l'ensemble des résultats de ce travail de thèse soulèvent par ailleurs des questionnements à plus long terme.

1. Activation et perte d'expression de Fxrα : du pareil au même ?

Nous montrons dans les articles II et III que le récepteur Fxrα est un acteur clé impliqué dans le contrôle de la stéroïdogenèse, en accord avec ce qui avait été publié par Volle *et al*. (2007). De façon surprenante, nous notons que l'activation de l'activité transcriptionnelle de Fxrα par le régime enrichi en acides biliaires ou son agoniste synthétique GW4064 (article II et Volle *et al*. 2007) abouti à un effet identique à la perte d'expression de Fxrα (modèle KO décrit dans l'article III), à savoir la répression de l'expression des gènes codant les enzymes stéroïdogènes (Star, Cyp11a1).

Ce cas de figure n'est pas isolé parmi les récepteurs nucléaires : le niveau d'accumulation de certains gènes cibles (Abca1, Star) des récepteurs nucléaires Lxrs est également connue pour être régulée de façon identique en l'absence des Lxrs et en réponse à leur activation transcriptionnelle par la présence de ligand (411)-(412). Cette apparente régulation identique n'est pas vraie pour tous les gènes, notamment pour Srebp1f codant Srebp-1c (411). Ces deux récepteurs (Lxr et Fxrα) appartiennent à la même classe de récepteurs nucléaires, et partagent un mode d'action similaire. Comme décrit dans l'introduction de ce manuscrit, ils sont fixés de manière constitutive sous forme d'hétérodimère avec Rxr sur le promoteur de leurs gènes cibles en l'absence de ligand. Ils sont associés à des co-répresseurs maintenant la chromatine dans un état répressif vis-à-vis de la transcription. La fixation du ligand sur le récepteur permet le relargage de ces co-répresseurs, et le recrutement de co-activateurs et de la machinerie transcriptionnelle qui permettra d'enclencher l'expression du gène cible. En ce basant sur ces modalités d'action, Wagner *et al*. (2003) (411) ont apporté une explication à ce phénomène en procédant à l'immunoprécipitation des co-répresseurs connus des Lxrs, *Nuclear receptor Co-Repressor* (N-cor) et *Silencing Mediator for Retinoic acid and Thyroid hormone receptors* (Smrt), sur le promoteur du gène Abca1 dans des macrophages issus de souris sauvages et Lxrαβ$^{-/-}$ traités ou non par un agoniste de ces récepteurs. Ils ont montré que Ncor et Smrt sont associés au promoteur d'Abca1 dans les macrophages issus de souris sauvages en l'absence de ligand ; cette association est significativement diminuée en réponse à l'ajout de ligand. De façon intéressante, ils ont montré une très faible interaction de ces co-répresseurs avec le promoteur d'Abca1 dans les macrophages issus de souris Lxrαβ$^{-/-}$ en condition basale, de même qu'en présence du ligand. L'activation de l'expression du gène codant Abca1 en réponse au ligand et en l'absence des Lxrs est donc le résultat de l'absence des co-répresseurs sur son promoteur.

Un phénomène équivalent pourrait se produire pour Fxrα. Celui-ci réprime la stéroïdogenèse indirectement en activant l'expression des répresseurs transcriptionnels Shp et / ou Dax-1 (Volle *et al*. 2007, article II). Notons par ailleurs que l'expression de Shp est augmentée chez les animaux Fxrα$^{-/-}$ par rapport aux animaux sauvages. Nous envisageons donc une stratégie identique à celle employée par Wagner *et al*. pour le gène codant Shp et / ou Dax-1 dans des cellules de Leydig primaires issues de souris sauvages et Fxrα$^{-/-}$. Nous avons choisi de cibler les co-répresseurs connus de Fxrα (Ncor (413), et l'histone déacétylase *Sirtuin 1* (Sirt1) (414)). Ceux-ci sont exprimés respectivement dans les cellules de Leydig *in vivo* et dans la lignée TM3 (415)-(416).

De façon intéressante, ce mode d'action apparaît gène et / ou type cellulaire dépendant, puisque Jmjd3 répond lui de façon opposée entre la perte de Fxrα in vivo et l'activation de Fxrα *in vitro*. Cette observation reflète toute la complexité des mécanismes mis en jeu par Fxrα pour la régulation de l'expression de ces gènes cibles, et souligne la difficulté d'anticiper les effets générés par l'activation ou l'inhibition de la signalisation Fxrα. Cela met par ailleurs en lumière l'importance des études pré-cliniques quant-à l'évaluation de l'efficacité et des effets secondaires d'un traitement utilisant un agoniste ou un antagoniste de Fxrα.

2. Diminution du nombre de cellules de Sertoli et augmentation de la production spermatique : une nouvelle vision de la physiologie testiculaire ?

Nous mettons en évidence une augmentation de la production de spermatozoïdes chez les animaux Fxrα$^{-/-}$ par rapport aux animaux sauvages (article III). Le rendement de la production spermatique est déterminé par le nombre de cellules de Sertoli, établi au cours de la période embryonnaire et du développement pubertaire : en effet, chaque cellule de Sertoli ne peut soutenir la différenciation que d'un nombre limité de cellules germinales. De façon tout à fait surprenante, les animaux Fxrα$^{-/-}$ présentent non pas une augmentation, mais une diminution du nombre de cellules de Sertoli à l'âge adulte. De plus, l'analyse GSEA révèle la répression d'une majorité des gènes enrichis dans les cellules de Sertoli chez ces animaux par rapport aux souris sauvages, à un âge (10 jours post-nataux) où le nombre de ces cellules n'est pas encore altéré. La perte de Fxrα aboutirait donc à une diminution du nombre de cellules de Sertoli, mais également à une altération de leurs fonctions. Dans ce contexte, comment expliquer que ce nombre limité de cellules de Sertoli, aux fonctions potentiellement compromises, puissent être capable de soutenir une spermatogenèse augmentée en terme de quantité ?

L'analyse GSEA met en évidence une petite proportion de gènes dont l'expression est augmentée chez les animaux Fxrα$^{-/-}$ par rapport aux animaux sauvages. *Nous souhaitons nous pencher plus particulièrement sur l'étude de ces gènes, dans le but de les relier à certaines fonctions des cellules de*

Sertoli qui pourraient être suffisantes pour le soutien de la différenciation germinale. Cette étude pourrait permettre de mieux comprendre les interactions s'établissant entre les cellules de Sertoli et les cellules germinales, et de hiérarchiser les différentes fonctions des cellules de Sertoli en lien avec le soutien de la spermatogenèse.

3. Fxrα : une cible potentielle de perturbateurs endocriniens ?

En lien avec le rôle central joué par Fxrα dans la synthèse des stéroïdes (Volle et *al.*, articles II et III) et le catabolisme hépatique des androgènes (article I), se pose la question de Fxrα en tant que cible de perturbateurs endocriniens. En effet, la définition donnée par l'OMS à un perturbateur endocrinien (PE) est ''une substance ou un mélange exogène altérant les fonctions du système endocrinien et provoquant par conséquent des effets néfastes sur la santé d'un organisme intact, ou sa descendance ou de (sous-)populations'' (*International Programme on Chemical Safety*, 2002).

Les agonistes et antagonistes de FXRα peuvent-ils dès lors être considérés comme des perturbateurs endocriniens à part entière ?

Le stigmastérol et la guggulstérone sont des antagonistes naturels de Fxrα. Le stigmastérol est présent notamment dans les huiles de colza ou de soja, administrées aux nourrissons prématurés dans le cadre de nutrition parentérale. La guggulstérone est utilisée, notamment en Inde pour le traitement de l'hypercholestérolémie (417). Les travaux de Hsu ont par ailleurs définis les insecticides cyfluthrin et bifenthrin comme des antagonistes sélectifs de Fxrα (418). Enfin, de part le rôle central joué par Fxrα dans le contrôle du métabolisme des acides biliaires, du cholestérol, des tryglicérides ou encore du glucose, des molécules synthétiques ciblant Fxrα ont été développé dans le cadre du traitement de pathologies métaboliques telles que la dyslipidémie, le diabète ou encore la stéatose hépatique non alccolique (NASH). Certaines de ces molécules sont d'ailleurs en cours de test clinique (419). *Nos résultats soulèvent donc la question de l'exposition à de telles molécules sur la mise en place, et le maintien de la fonction de reproduction masculine.*

4. La perte d'expression de FXRα : une prédisposition génétique au développement du cancer du testicule ?

Le cancer du testicule est le cancer le plus fréquemment diagnostiqué chez l'homme jeune (15-35 ans) (420). Il intervient donc au cours des années propices à la paternité et peut avoir des répercussions irréversibles sur la fertilité des patients, ce qui en fait un problème sanitaire non négligeable. L'étiologie de ce cancer, probablement multi-factorielle, reste à ce jour peu connue. Cependant, l'augmentation constante de l'incidence de cette pathologie (6.4/100.000 hommes en France) dans les pays industrialisés depuis plusieurs décennies, ainsi que les disparités régionales

observées, laissent présager de l'implication de l'exposition à des molécules environnementales couplée à des prédispositions génétiques dans la survenue de ce type de cancer.

Les cellules germinales sont à l'origine de l'immense majorité (95%) des tumeurs testiculaires (421). Les tumeurs germino-testiculaires sont classées en deux catégories en fonction de leur composition cellulaire : les séminomes et les non-séminomes. Elles dériveraient en majorité de carcinomes *in situ* (422), caractérisés par la présence dans les tubes séminifères de grandes cellules plurinucléées, similaires aux gonocytes présents dans le testicule fœtal, et exprimant de façon spécifique certains facteurs de cellules pluripotentes tels que oct3/4 ou nanog (423)-(424). Ces données suggèrent une origine fœtale au cancer testiculaire. Cependant, celui-ci ne survient dans la majorité des cas qu'à partir de 15 ans, soit au moment de la puberté, cette étape s'avère donc être une autre période critique pour le développement de cette pathologie.

Les résultats de l'analyse par RNA-seq du testicule de souris mâles sauvages et Fxrα$^{-/-}$ de 10 jours ont révélé une altération de l'expression de nombreux gènes (article III). Parmi eux ont été identifiés plusieurs gènes impliqués dans le développement embryonnaire (gènes homéotiques Hox a5-6, b1-3-6), ainsi que des marqueurs de cellules pluripotentes (Oct3/4, Nanog). Ces résultats pourraient suggérer la persistance chez les souris Fxrα$^{-/-}$ de cellules germinales embryonnaires à l'origine du carcinome *in situ* testiculaire chez l'Homme. Par ailleurs, nous avons noté au moment du développement pubertaire, une augmentation la prolifération et de la survie des cellules germinales chez les animaux Fxrα$^{-/-}$ par rapport aux animaux sauvages. Enfin, des données préliminaires obtenues grâce à notre étroite collaboration avec l'équipe du Dr.Rajpert-De Meyts (CHU de Copenhague, Danemark) ont permis de démontrer une diminution significative de l'accumulation des messagers codant FXRα dans les échantillons de biopsies testiculaires tumorales humaines par rapport aux échantillons sains.

L'ensemble de ces résultats suggère que l'invalidation génétique du gène codant pour Fxrα pourrait prédisposer le testicule à la formation de tumeurs germinales. Cependant, les mâles Fxrα$^{-/-}$ ne développent pas de cancer testiculaire, signifiant que la perte de Fxrα n'est pas suffisante à elle seule pour provoquer le développement d'une telle pathologie. Le cancer étant une pathologie multi-factorielle, nous avons émis l'hypothèse selon laquelle la perte de Fxrα pourrait constituer un terrain favorable au processus de tumorigenèse testiculaire en réponse à une exposition environnementale. L'intérêt et la pertinence de ce questionnement ont été appuyés par deux financements obtenus récemment par l'équipe à ce sujet (Plan Cancer 2014 et bourse de soudure de thèse par la Fondation ARC 2015).

En lien avec les études épidémiologiques, les molécules environnementales choisies sont les suivantes : le bisphénol A (Plan Cancer), dont l'impact délétère sur la physiologie testiculaire est bien décrit (425), et le pesticide fenvalerate (projet ARC). En effet, bien que controversées, des études épidémiologiques ont mis en évidence une augmentation de l'incidence de troubles de la fertilité et de cancers testiculaires dans les catégories socio-professionnelles utilisant les pesticides (atrazine, fenvalerate, malathione...), tels que les agriculteurs, par rapport au reste de la population (426). Nous avons ciblé plus particulièrement le fenvalerate car ce dernier a un effet pro-apoptotique sur les cellules germinales au stade méiotique (427). Ainsi, nous émettons l'hypothèse selon laquelle l'hyper-prolifération germinale, observée chez les mâles Fxrα$^{-/-}$ de 15 jours, couplée à un tel blocage de la différenciation pourrait conduire au processus tumoral.

a) Perte d'expression de Fxrα et exposition environnementale : un nouveau modèle de tumorigenèse testiculaire ?

Les facteurs impliqués dans la survenue et le développement des tumeurs germinales sont peu connus. L'étude de ces mécanismes est rendue difficile par le manque d'un modèle animal développant de telles tumeurs. En effet, l'apparition spontanée de tumeurs germinales est très rare chez les animaux de laboratoire (428), et lorsqu'elles surviennent, elles ne partagent pas les caractéristiques des tumeurs observées classiquement chez l'Homme (423)-(429). Dans le cas où nos modèles développeraient des carcinomes *in situ* et des lésions séminomateuses ou non séminomateuses classiques, ils seraient donc pertinents pour l'identification d'acteurs moléculaires impliqués dans l'initiation et la progression des tumeurs germinales, et ouvriraient la voie pour le développement de nouvelles molécules thérapeutiques qui se substitueraient à l'ablation chirurgicale du testicule affecté.

L'analyse de l'implication potentielle de Fxrα dans la progression tumorale sera également approchée in vitro *en analysant l'impact de la répression de la signalisation associée à FXRα (ARN interférence, antagonistes) sur les caractères tumoraux (prolifération, apoptose, migration, invasion, expression de marqueurs d'agressivité...) de lignées séminomateuse (TCam-2) et non séminomateuses (NTERA-2 et NCCIT) qui expriment FXRα.*

b) Quelles applications chez l'Homme ?

Afin de valider la pertinence de notre hypothèse chez l'Homme, nous avons établi une collaboration avec l'équipe de Béatrice Fervers (Centre de Recherche en Cancérologie, Lyon). Celle-ci a pour but de *générer une cohorte de patients ayant développé une tumeur germinale, sur laquelle nous rechercherons la présence de polymorphismes dans la région génomique du gène codant FXRα, en*

portant un intérêt plus particulier aux sujets étant exposés de part leur profession ou leur mode de vie à des molécules chimiques environnementales.

L'ensemble de ce travail de recherche doctoral définit Fxrα comme un acteur clé de la physiologie et la physiopathologie du testicule. Nos résultats montrent qu'il contrôle en effet la fonction physiologique des cellules de Leydig, de Sertoli et des cellules germinales, par des mécanismes moléculaires qu'il reste à préciser. Ces travaux posent par ailleurs la question de l'impact éventuel de l'utilisation chronique d'agonistes et d'antagonistes de Fxrα, notamment dans le traitement de pathologies métaboliques telles que la dyslipidémie ou le diabète, sur la fonction de reproduction.

ANNEXES

Is spermiogenesis the critical step for answering biomedical issues ? *(review)*

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Male fertility

Is spermiogenesis the critical step for answering biomedical issues?

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Regarding male fertility, biomedical issues have opposite goals to treat infertility or develop contraceptive drugs. Recently, the identification of the molecular mechanisms involved in germ cell differentiation suggest that spermiogenesis has to be put at the crossroad to reach these goals.

Concerning fertility issues, citizens in our modern world are schizophrenic. On one side, couples have the possibility to control conception; and on the other side, more and more couples suffer from the misfortune of being infertile. These two societal problems lead to intensive research and conflicting government policies. However, these opposing goals rely on a better understanding of germ cell differentiation.

Keywords: male, infertility, contraception, spermatogenesis, spermiogenesis

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Male Contraception

In our society, contraception is a widely discussed topic among couples. Contraception is used by couples for family planning purposes. It is also important to reduce the number of deaths and diseases in women who have unwanted pregnancies.[1] This is why governments have contraceptive programs in place to support women with undesired pregnancies. Regarding contraception, it is rarely a question for men since the only available methods are the use of condoms and vasectomy.[2] The lack of male contraception is also because for men, the daily production of up to 100 million spermatozoa is a much bigger hurdle to deal with vs. just one ovum every month in women. For male contraception, much research and efforts have been focused on hormonal approaches.[3,4] Indeed, testicular physiology is mainly regulated by the hypothalamus-pituitary axis, which produces gonadotropins that control the endocrine (steroidogenesis) and exocrine (gamete production) functions of the testis.[5] Both are interconnected as germ cell survival is dependent of the androgen concentrations.[6] Thus, hormonal strategies are used to block spermatozoa production. However, a long-term impact of hormonal-based contraceptives is not known, which may expose men to health risks as demonstrated by the adverse effects of endocrine disrupters.[7] There are also efforts in the field to block gamete production via spermatogenesis in which undifferentiated spermatogonia are expanded by consecutive cycles of mitosis, to be followed by germ cell differentiation and meiosis, and with the haploid spermatids, undergo spermiogenesis to become spermatozoa.

Another interesting question about male contraception is the accessibility of contraceptive drugs to target cells. This is important in the testis since the testis is an immune-privileged organ and it is equipped with sophisticated structures to minimize toxicity in order to protect developing germ cells. Indeed, in the testis, the blood-testicular barrier (BTB) is a structure in the seminiferous epithelium, essential for spermatogenesis.[8] This poses a major obstacle in delivering male

contraceptive drugs to meiotic and post-meiotic cells that are located behind the BTB. It has been established that only a small fraction of the drugs (1%) administered to adult rats via gavage could reach the testis.[9]

Thus, a better understanding of the BTB and a novel approach to disrupt the BTB specifically could facilitate male contraceptive development. In this context, adjudin is a potent male contraceptive. It breaks down cell/cell adhesion between Sertoli and germ cells in the seminiferous epithelium, causing transient infertility.

Besides the BTB, testis also express many drug transporters that are capable of pumping drugs and/or toxicants, including male contraceptives out of the testis via efflux drug transporters.[10-12] More important, these efflux and influx drug transporters are also found in spermatogonia and early spermatocytes, which are located outside the BTB. In short, a better understanding of drug transporters and the BTB are critical to develop male contraceptives.

Inhibition of germ cell proliferation. Spermatogonia are the forerunners of all spermatogenesis, and some of these cells are also the "spermatogonial stem cells" of the germ cell lineage. Targeting spermatogonia is risky, as it could irreversibly affect daughter cells[13] and progeny even after the contraception treatment has been stopped. In addition, altering the proliferation of these cells could result in tumor development.

Meiosis as the target step for contraception. The second step to focus on could be meiosis. In the last decade, knowledge about meiosis has increased. We will not give an exhaustive review, but some points are of interest for contraception.

The meiotic step is sensitive to hormonal status, as demonstrated in rodent transgenic models[6] and by endocrine disrupters.[7] This step of spermatogenesis could be targeted by hormonal contraception. However, such an approach may affect secondary sexual characteristics, as the hypothalamic-pituitary axis will be altered.

The entry to and progression through meiosis are under the control of the retinoid signaling pathways.[14,15] Up until puberty, cytochrome Cyp26b1 maintains low concentrations of retinoids. At puberty, the retinoid levels increase and induce the expression of stimulated by retinoid gene-8 (Stra8), which allows germ cells entry to meiosis.

In contrast, fibroblast growth factor 9 (FGF9) suppresses entry to meiosis. FGF9 decreases the sensitivity of germ cells to retinoids. In addition, FGF9 signaling preserves the pluripotency of germ cells and promotes a male fate during embryonic development.

Retinoid and FGF9 act in concert to control mammalian germ cell sexual fate commitment.[16] Developing drugs to modulate these pathways could be of interest for contraceptive issues.

During meiosis, homologous recombination allows the reciprocal exchange of genetic material between parental genomes and ensures proper chromosome segregation during the first meiotic cell division.[17-19]

Spo11, a meiosis-specific protein, introduces double-strand breaks on chromosomal DNA and sets up meiotic recombination. The spermatocytes of Spo11-/- mice fail to synapse chromosomes and progress beyond the zygotene stage of meiosis.[20] The Ataxia telangiectasia-mutated (ATM) kinase, activated by DNA damage, triggers checkpoint signaling and promotes DNA strand break repair in order to pass meiosis.[21] In addition, many transgenic rodent models show altered meiosis because of a failure in chromosomal synapsis. Similarly, TEX15-deficient spermatocytes exhibit a failure in chromosomal synapsis.[22] Zip4h(-/Y) mutant mice present a delay in meiotic double-strand break repair and decreased crossover formation.[23]

In addition, studies have demonstrated the importance of germ cell-specific epigenetic marks in the entry to and progression of meiosis.[24] Interestingly, mice with a loss-of-function mutation in H3K9 histone methyltransferases are sterile, as the germ cells undergo apoptosis at the pachytene stage.[25,26] Several proteins possess H3K9 methyltransferase activity. Suv39h1, Suv39h2 and G9a can perform H3K9 dimethylation, whereas only G9a performs H3K9 monomethylation.[27]

MicroRNA and small RNA also play important roles in germ cell differentiation and transmission to following generations.[28-30] The correct spatial and temporal expression of germ cell-specific genes is essential to produce functional spermatozoa.[31] Dicer 1, an RNase III endonuclease, is essential for the biogenesis of microRNAs (miRNAs) and endogenous small-interfering RNAs (endo-siRNAs). It also degrades toxic transposable elements. Early ablation of Dicer1 at the onset of male germ cell development leads to infertility caused by multiple cumulative defects. Alterations such as delayed progression of spermatocytes to prophase I and increased apoptosis were observed in the first spermatogenic wave, resulting in a reduced number of round spermatids.[32]

In the future, it may be proposed to develop drugs targeting these key processes of meiosis. However, it must be ensured that these drugs will block 100% of the germ cells at meiosis; if some pass this step with incomplete DNA repair or abnormal epigenomic pattern, it could impact the development of the offspring.

Spermiogenesis as the last testicular step on which to focus. Spermatozoa production may also be blocked at spermiogenesis, the stage in which spermatocytes become haploid spermatids. During this stage, much of the cytoplasm is removed through phagocytosis by the Sertoli cells.[33] There is also a condensation of the genome following the replacement of histones by protamines.[34] Several post-translational modifications occur on histones during spermatogenesis. Histone H4 hyperacetylation is associated with histone removal; however, the exact mechanisms are still unclear. Finally, acrosome formation and the establishment of the flagellum lead to spermatozoa.[35]

Recently, Matzuk et al. characterized the reversible inhibitory impact of a small molecule, 4-(4-chlorophenyl)-2,3,9-trimethyl-1,1-dimethylethylester-6H-thieno(3,2-f)(1,2,4)-triazolo(4,3-a)-(1,4)diazepine-6S-acetic acid (JQ1), on mouse fertility.[36] The JQ1 molecule interacts with and is an inhibitor of the bromodomain and extra-terminal (BRDT) subfamily of epigenetic reader proteins.[37,38] BRDTs are expressed during the later stages, from pachytene spermatocytes to spermatids. BRDTs colocalize and interact with acetylated H4

in elongating spermatids,[39] and they are involved in the marked chromatin remodeling during spermiogenesis that leads to the histone-protamine transition and nucleus condensation.

JQ1 does not have an impact on testosterone levels, which is important for the preservation of other male characteristics. In addition, it does not alter the proliferative properties of spermatogonia. Consistent with the cellular-restricted expression of Brdt1, JQ1 must act on spermatocytes and spermatids, leading to a decrease in the number of round spermatids and spermatozoa. Additionally, it affects spermatozoa quality, as analyzed by their motility.

A Long Way to Go and Many Questions to Answer Before Using Such a Contraceptive Molecule

(1) The contraceptive effect of JQ1 is reversible, but mice only recover fertility 4–6 mo after the end of treatment. The duration of spermatogenesis in mice is 35 d, compared with 74 d in humans. Six months equates to six cycles of spermatogenesis in mice, so the dose and treatment length in humans must be defined, as six cycles of spermatogenesis in humans takes longer than a year to complete.

(2) The chromatin changes associated with the use of the JQ1 molecule must be analyzed carefully to avoid harmful effects on the offspring.

(3) In their study, Matzuk et al. did not test the long-term impact of such treatment. Indeed, male contraception would imply a longer time frame than the one tested.

(4) During his reproductive life, a man may need to use such a molecule at various periods, raising the question of whether the recovery time will be the same after multiple exposures.

(5) There are several homologous members in the BRDT family. Further studies will be necessary to ensure the specificity of JQ1 on chromatin modifications during spermiogenesis. For example, BRD4 and BRDT1 show high similarity even on the JQ1-interacting domain, and BRD4 is associated with several pathologies including cancers.[40,41]

Male Infertility

Infertility affects approximately 15% of couples. Males account for 40–50% of the cases, either alone or in combination with female pathologies.[5] The incidence of male fertility disorders is continuously increasing and has been linked to multiple factors, including genetic and environmental factors.[42] Several epidemiological studies associate environmental factors and toxic chemicals such as endocrine disruptors (phthalates or bisphenol-A) to male infertility.[43,44] In parallel, at least 40% of people undergoing anticancer treatments have impaired reproductive function. Chemotherapy or radiation may impair fertility, sometimes irreversibly.[45] The fertility side effects of these treatments should be considered before beginning the treatment. However, for some patients, it is not possible to preserve the gametes, such as in children who contract cancers before puberty. In addition, it might be risky to preserve the spermatozoa of men with metastatic diseases, as there is no assurance that the germ cells have not been altered. In these cases, it may be safer to find other alternatives to restore the male fertility.[46]

Infertility affects millions of people worldwide. For many people, the only possibility is to use assisted-reproductive technologies (ART). In vitro fecundation and intra-cytoplasm injection are long and difficult processes at both physical and psychological levels.[47] Furthermore, biopsy is an invasive approach and is associated with an increased risk to develop testicular cancer.[48] In some infertility cases, however, it is not possible to find either spermatozoa or elongated spermatids in testicular biopsies. In such cases, people may decide not to be the biological father of their child, which can be difficult for both the father and the children once they get older.[49,50]

Other alternatives should be able to overcome these difficulties. The current technologies have reached their limits, leading many researchers to make efforts in developing in vitro approaches to differentiate germ cells. However, it is difficult to differentiate germ cells that are able to fertilize oocytes. The differentiation of germ cells requires specific factors that allow pluripotent cells to enter a specific lineage. Another critical step is for germ cells to pass meiosis. At this point, the haploid cells will condense their nuclei and change their shape while forming the acrosome and the flagella, essential apparatus for motility and fertilization of the oocytes.

To circumvent these difficulties, we need to improve our knowledge of germ cell differentiation.

Protocols for the differentiation of germ cells went through several experimental steps before gaining some efficiency and were mostly performed using fetal cattle male germ cells.[51] However, as fetal germ cells from humans are difficult to obtain, such approaches are hardly feasible in human clinics. Other groups managed to differentiate haploid cells from spermatogonial stem cells, but the experimental protocol, which uses fluorescence-activated cell sorting (FACS) technology to select cells that will be able to give haploid cells, is difficult to perform.[52] Human-induced pluripotent stem cells can produce haploid germ cells.[53] The experimental protocol requires culture for 10 wk and the use of FACS to obtain haploid cells. However, the percentage of selected cells is low, as only 1–2% of the cultured cells are haploid.

All these data have provided many clues to better understand spermatogenesis. A recent study by Easley et al.[54] has led to a jump in the capacity to differentiate spermatogenic cells. The authors described how to obtain haploid spermatogenic cells from human foreskin fibroblasts without any genetic manipulation. Using a specific cell culture protocol, they have been able to differentiate these cells into post-meiotic round spermatids in a very short amount of time, only 10 d. In addition, this protocol is more efficient, as 4–5% of human pluripotent stem cells give haploid cells.

Although the study from Easley et al. produced some interesting results, various points need to be clarified. Ten days seems too short period to produce haploid cells compared with the classical length of spermatogenesis in human (74 d). It must be confirmed that the spermatids have been correctly differentiated. Another striking point is that a longer culture time

did not allow the authors to obtain more haploid cells; in fact, the percentage even decreased after 20 d of culture, suggesting that the other germ-like cells (VASA+) do not have the capacity to differentiate into haploid cells.

However, this protocol may allow researchers to study events during early spermatogenesis from human primordial stem cells (hPSCs) to spermatogonial stem cells (SSCs) and to characterize the events associated with spermiogenesis to the round spermatid stage. If there is a long way to go, such results highlight the need to increase our knowledge of spermatogenesis to be able to transplant germ cells that will differentiate in vivo in functional spermatozoa in the future.

In addition, the cells failed to perform complete spermiogenesis. This study highlights the complexity of such differentiation and identifies some critical missing factors in this experimental protocol. This research highlights the potential need for supporting cells, namely Sertoli cells, for completing spermatogenesis in vitro. Sertoli cells play a supportive role to germ cells and maintain spermatogenesis (Cheng et al., 2010). They form the hemato-testis barrier that isolates the germ cells from blood components, particularly immune mediators.[55] It is worth noting that these functions might not be essential for the in vitro differentiation of germ cells. The role of Sertoli cells as the only source of nutrients and growth factors for germinal cells is bypassed in vitro by adding growth factors, serum and other supplements to the medium. However, the critical role of Sertoli cells of removing germ cell cytoplasm might be missing. Co-cultures of germ cells with Sertoli cells may be helpful to help human pluripotent fibroblasts to become haploid spermatids.

Buganim and collaborators managed to differentiate fibroblasts into embryonic Sertoli-like cells that are able to support germ cell survival in vitro.[56] The addition of such cells could help to complete germ cell differentiation. This idea is supported by data showing that in vitro SSC lines or any isolated SSCs can perform full spermatogenesis.[57] These data were obtained using an approach in which SSCs were transplanted into organ cultures. The obtained haploid cells (elongated spermatids and flagellated sperm) gave rise to healthy offspring through micro-insemination. Using foreskin fibroblasts to perform co-culture of Sertoli-like cells and germ cells may be a major advance toward helping male infertility.

A Long Way to Go and Many Questions to Solve Before Differentiating Fully Competent Germ Cells

(1) Although the potential use of human foreskin fibroblasts opens a new field of research in the production of differentiated germ cells, the quality of the germ cells must be controlled before using such cells for ART. It will be necessary assess the criteria used to define differentiation. Is differentiation only morphological? Is it the correct expression of specific cell-type markers (protamine, acrosin, etc.)? Is it a specific epigenetic pattern (DNA methylation; histones/protamines)?

(2) Further tests will be needed to assure that these haploid germ cells can, at least in vitro, give rise to embryos.

(3) Once in vitro differentiation of efficient germ cells has been achieved, society will also have to address the bioethical challenges.

(4) The use of animal models also represents a tool for exploring the root causes of male infertility. Deriving hPSCs from infertile men will allow identification of where spermatogenesis arrests. It will be important to pre-clinically evaluate whether these in vitro-generated gamete forerunners have reproductive capacities in vivo. Re-implantation studies and analyses of embryo development in utero will also be necessary. As research on humans should be avoided for ethical reasons, inter-species studies will be required. Such approaches have already been performed and have succeeded between goats and mice. The use of animal models will be of major importance, as there is a large source of mouse models with altered spermatogenesis, altered Leydig or altered Sertoli functions. Approaches with transgenic models may be helpful in analyzing the cell/cell communication and paracrine factors involved in complete spermatogenesis.

Focusing Research on a Common Target to Improve ART and Contraceptive Issues?

During recent decades, there has been increasing interest in biomedical issues involving infertility and contraception.

In addition, ethnical issues could alter contraceptive approaches.[58] For example, the use of condoms is associated with sex outside marriage in Africa, inhibiting their use between married couples.

Vasectomy is, of course, an irreversible method of contraception under male control. The prevalence of vasectomy varies widely around the world. Some couples that choose sterilization cite problems with other contraceptive methods. In addition, couples that choose sterilization are more likely to be older, to be married and to have children. In some countries (for example in Latin America and the Caribbean), couples that choose sterilization are more likely to be of higher socioeconomic status, while in others (for example in India and Bangladesh), they are more likely to be of lower socioeconomic status.

Attitudes toward male condoms and new contraceptive methods varied markedly according to cultural background. One study reported that men from South Africa, China and Hong Kong were less enthusiastic about hormonal contraception for men,[59] while another study reported significant cultural variations in the acceptability of hormonal contraception for men.[60] Educated men were more likely to find the idea acceptable. Those opposed to contraception either in general or because of their religious beliefs were likely to approve of male methods.

Masculinity is rarely evoked about contraceptive approaches when male alterations are involved in the decrease of fertility, particularly in the context of endocrine. If testicular biopsies can help in obtaining spermatozoa with infertility due to altered germ cell production, other approaches need to be developed. Indeed, a biopsy is an invasive approach and is associated with an increased risk for developing testicular cancer.[48] One might think that to transplant spermatogonia differentiated in vitro from fibroblasts might allow a man to be the biological father of his children. Of

course, such transplantation approaches could be successful only when the origin of the default is within the germ cells. If the problems come from endocrine functions or Sertoli cells for example, transplantation of spermatogonial stem cells will not have any chance to succeed. If the alterations (mutation, epigenetic information) are intrinsic to germ cells, however, transplantation could be of major interest.

Concluding Remarks

To reach these two opposite objectives of reproductive biology, the common point seems to better understand the key steps involved in spermatogenesis. Such knowledge is essential to identify the clues necessary to successfully differentiate germ cells in vitro and to identify the best contraceptive molecules.

Furthermore, obtaining in vitro differentiated spermatozoa will be less invasive than performing biopsies. The work of Easley et al. thus opens new perspectives, as the authors have been able to differentiate germ cells from pluripotent fibroblasts. However, it still difficult to differentiate germ cells up to spermatozoa. This difficulty to pass spermiogenesis suggests that it is a key step of germ cell differentiation.

A study with the opposite purpose of generating a new contraceptive approach also highlights the importance of this step of spermatogenesis. The use of JQ1 arrests germ cell differentiation at haploid step, as shown by the decreased number of spermatids.

Both studies identify chromatin remodeling as a critical step. Epigenetic modifications are of major importance for development, as epigenetic changes can lead to embryonic death. This was shown in rodent models invalidated for genes encoding DNA-methyltransferases.[61] In addition, alterations in the epigenetic pattern (DNA or histone modifications) correlate with an increased incidence of pathologies such as cancer.[62] Increased knowledge of germ cell differentiation is necessary to avoid or to minimize impacts on the offspring, even after several generations. To restore fertility using in vitro-differentiated haploid cells, a correct epigenetic pattern will be necessary to achieve the best rate of embryonic development in vitro and to reduce spontaneous abortions after re-implantation in females and the incidence of severe pathologies in the progeny.

The role of epigenetics in male gametes must also be evaluated. Indeed, ART approaches may lead to epigenetic modifications in the embryos developed in vitro.[63] Although epigenetics has not clearly been shown to have an impact on progeny development, the fact that epigenetic modifications result in developmental defects that lead to embryonic death, which could contribute to the low efficiency of ART approaches (approximately 20% success rate) cannot be excluded. A recent study suggests that epigenetic modifications during ART are normally corrected in the germ line by epigenetic reprogramming and, thus, not spread to following generations.[64] However, such impacts still need to be further studied, as rare epigenetic disorders such as Beckwith-Wiedemann syndrome or Angelman syndrome seem higher.[65,66]

Likewise, it will be necessary to control for the quality of germ cells that will be generated after the arrest of the temporary contraception in men. This includes morphological aspects as well as classical quality criteria such as mobility and capacity to fecund. The emergence of the importance of epigenetic status in haploid germ cells indicates that these criteria will also need to be taken into consideration.

All these studies on spermatogenesis will help in developing new strategies for contraception and for new approaches for restoring fertility. From all these recent data, the identification of the molecular mechanisms involved in such a complex process will give some hints and contribute to the emergence of a new field of biomedical research. Finally, these studies suggest that spermiogenesis must be placed at the crossroads to reach the goals of the two main issues of biomedical reproductive biology, namely male contraception and male infertility.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Farnesoid X receptor alpha: a molecular link between bile acids and steroid signaling ? *(review)*

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Farnesoid X receptor alpha: a molecular link between bile acids and steroid signaling?

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Abstract Bile acids are cholesterol metabolites that ha ve been extensively studied in recent decades. In addition to having ancestral roles in digestion and f at solubilization, bile acids ha ve recently been described as signaling mol - ecules involved in many physiological functions, such as glucose and energy metabolisms. These signaling pathways involve the acti vation of the nuclear receptor f arnesoid X receptor (FXRα) or of the G protein-coupled receptor TGR5. In this re view, we will focus on the emer ging role of FXRα, suggesting important functions for the receptor in steroid metabolism. It has been described that FXR α is expressed in the adrenal glands and testes, where it seems to control steroid production. FXR α also participates in steroid catabolism in the li ver and interferes with the ster - oid signaling pathw ays in tar get tissues via crosstalk with steroid receptors. In this re view, we discuss the potential impacts of bile acid (BA), through its interactions with ster- oid metabolism, on glucose metabolism, se xual function, and prostate and breast cancers. Although several of the published reports rely on in vitro studies, they highlight the need to understand the interactions that may af fect health. This effect is important because B A levels are increased in several pathophysiological conditions related to li ver injuries. Additionally, BA receptors are tar geted clinically using therapeutics to treat liver diseases, diabetes, and cancers.

Keywords FXRα · Bile acid · Steroids · Physiologic functions

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Bile acids

Biosynthesis and physico-chemical function

Bile acids (BAs) are the main constituent of bile. B As are present in the digestive tract during a meal and ensure solu- bilization and emulsiȧcation of fat, thus helping digestion [1]. They are produced in the liver from cholesterol through a series of enzymatic modiȧcations. There are two different synthesis pathways that share common enzymes. The ȧrst, named the classical pathw ay, involves the P450 CYP7A1 and CYP8B1 c ytochromes, among others. The alternate pathway involves cytochromes CYP27A1 and CYP7B1. Both pathways result in the production of the so-called pri- mary BAs cholic acid (CA) and chenodeoxycholic (CDCA) [2]. Before being e xcreted by the hepatoc ytes, BAs are, in part, combined with amine residues (glycine or taurine) leading to the production of bile salt-, tauro-, or glyco-con- jugates. Primary BAs and their conjugates are stored in the

gallbladder and are discharged during a meal into the duodenum to facilitate the digestion of fats and their passage through the enterocyte barrier.

In the ileum, BAs are partially deconjugated and are modiûed by enzymes of the intestinal Ęora [3]. These transformations lead to the synthesis of secondary BAs. Thus, deoxycholic acid (DCA) and lithocholic acid (LCA) are derived from CA and CDCA, respectively.

In the ileum and colon, the majority of BAs (95 %) are reabsorbed for recycling in the liver. Thus, the newly synthesized BAs will again be excreted during several rounds of digestion. This recycling mechanism, named enterohepatic circulation, involves a system of ůnely regulated carriers to maintain the homeostasis of BAs and cholesterol from which the BAs originate [4].

In addition to this mechanical function, BAs have been described as molecules that signal through two receptors: the nuclear farnesoid X receptor alpha (FXR α; NR1H4) and the membrane receptor TGR5 (GPBAR1, G protein-coupled bile acid receptor).

Here, we will focus on the potential involvement of FXRα and bile acids on steroid metabolism.

The nuclear receptor of BAs: FXRα

FXRα is a member of the nuclear receptor family [5]. This receptor was isolated from mouse livers in a screen of proteins searching for proteins that interact with the receptor of 9-cis retinoic acid (RXR) and thus was previously named RXR-interacting protein 14 (RIP14) [6]. It was renamed FXRα because it was shown to be activated by farnesol, an intermediate of the mevalonate pathway. FXRα regulates transcription through heterodimerization with RXR, and binds speciůc sequences on the promoter of target genes, named the FXR-response elements (FXREs), to regulate transcription. These sequences are composed of two copies of a six-nucleotide sequence (AGGTCA) that are arranged as inverted repeat motifs separated by one base (IR-1) [7]. Other FXREs have been described, including IR0, IR8 (separated by zero or eight base pairs, respectively), eR8 (an everted repeat motif) and DR1 (a direct repeated motif), but these response elements have a lower afůnity than IR-1. If the FXRα/RXR heterodimer enhances transcription, it seems as FXRα can also repress transcription through potentially negative FXREs [8–10].

FXRα has also been shown to bind to certain genes as a monomer or a homodimer on negative FXREs [11]. These mechanisms are not yet fully understood.

FXRα/RXR is a permissive heterodimer, as ligands of both partners can synergize to regulate the transcription of target genes. In 1999, BAs were identiůed as ligands of FXRα [12–14]. This led to the renaming of FXRα as a "bile acid receptor" (BAR). The preferred ligands of FXRα are CDCA and its conjugated derivatives [15, 16]. Different bile acids have different potencies in regard to the activation of FXRα. The potencies are as follows, in decreasing order: (1) CDCA, (2) DCA, (3) LCA, (4) CA [13].

Human and mouse genes encode four isoforms of FXRα: FXRα1 (RIP14-2), FXRα2, FXRα3 and FXRα4 (RIP14-1) [17, 18]. The mouse FXRα gene is located on chromosome 10 C2, and the human FXRα gene is located on chromosome 12q23.1. These genes are composed of 11 exons and ten introns. The isoforms result from two alternate promoters that initiate transcription at either exon 1 or exon 3 [17, 19]. The alternative promoters at exon 1 or exon 3 regulate the expression of FXRα1 and FXRα2 or FXRα3 and FXRα4 transcripts, respectively. The FXRα3 and FXRα4 isoforms possess longer N-terminal regions than do FXRα1 and FXRα2. The isoform differences could impact the eůciency of the "activation function 1 domain" (AF-1) for interacting with cofactors. In the FXRα 1 and 3 isoforms, exon 5 is differentially spliced compared to FXRα 2 and 4. This alternative splicing event results in the addition of four amino acids (MYTG) adjacent to the DNA-binding domain in the hinge domain. The four FXRα isoforms present a degree of speciůcity at the mRNA level, which affects protein structure. However, all isoforms contain the classical domains of nuclear receptors, including the dimerization interface, the ligand binding domain, the DNA-binding domain, and the ligand-dependent activation function (AF-2) domain at the C-terminus. Indeed, the isoforms indicate classical activation by RXR and FXRα agonists, but could differentially regulate the expression of target genes in vitro [19, 20, 21]. Moreover, the isoforms are expressed in a tissue-speciůc manner.

The heart and adrenal glands express only FXRα1 and FXRα2. These isoforms are expressed at low levels in the lung and white adipose tissue. FXRα3 and FXRα4 are expressed in the kidney and stomach [22]. Volle et al. [23] showed the expression of FXRα in the testis, speciůcally in the interstitial compartment. This result was supported by a further study on testicular cell lines [24]. In humans, Bishop-Bailey et al. [25] showed expression of FXRα in biopsies of cardiac muscle, the small intestine and the adrenal glands. The liver and adrenal glands express FXRα1 and FXRα2 exclusively, while the kidney and the colon express FXRα3 and FXRα4. All four isoforms are found in the small intestine and duodenum. FXRα is detected in human immune cells, peripheral blood mononuclear cells and subsets of lymphocytes and monocytes [26, 27].

These expression patterns of FXRα suggest that it might have major physiological roles. The use of a mouse model lacking the gene encoding *FXRα* ($FXR\alpha^{-/-}$) highlights the involvement of *FXRα* in many physiological functions (digestion, immunity) and diseases, such as diabetes and cancers [28]. The ůrst described roles of FXRα were the

regulation of the enterohepatic cycle and the regulation of BA biosynthesis [29]. *Fxrα*$^{-/-}$ mice exhibit high plasma concentrations of BAs, highlighting the critical role of FXRα in the repression of *Cyp7a1*, which codes for a key enzyme in BA biosynthesis. At the molecular level, this pathway involves several members of the nuclear receptor superfamily, such as SHP (small heterodimer partner), LRH1 (Liver receptor homolog-1), and LXR α (Liver X receptor) [30, 31]. In parallel, FXRα protects the liver from the toxic effects of the accumulation of BAs, promoting the excretion of BAs into the bile by the transcriptional induction of speciȧc transporter *Bsep* (bile salt export pump) [32]. *Fxr*$^{-/-}$ mice consistently show decreased excretion of BAs in the digestive tract [29]. In the intestine, FXR α induces the expression and secretion of ȧbroblast growth factor 15/19 (FGF15/19) into the portal circulation. After binding with the ȧbroblast growth factor receptor 4 (FGFR4) in the liver, FGF15/19 represses the enzymes of BA synthesis [33].

FXRα is also involved in the control of lipid and carbohydrate metabolism [11]. Its action in the liver limits triglyceride production through the repression of genes such as stearoyl coenzyme A desaturase [34]. Consistently, *Fxrα*$^{-/-}$ mice show high plasma triglyceride concentrations. In addition, FXRα also controls glucose metabolism through the regulation, in the liver, of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase genes encoding key enzymes of gluconeogenesis and glycogenolysis [35].

Crosstalk between bile acids and steroid metabolism

In addition to the primary roles of bile acids, many recent studies have reported potential connections between bile acids and steroid metabolism.

It has recently been demonstrated that steroids can control bile acid homeostasis. For example, long-term therapy with glucocorticoids (GC) presents several limitations due to side effects such as hyperglycemia or insulin resistance. Cholestasis remains a major side effect. BA serum levels are correlated positively with serum GC concentrations in humans. Increased GC levels in Cushing's patients were associated with elevated BA levels [36]. Furthermore, hepatic GC receptor deȧciency in mice resulted in a reduction of the hepatic BA pool in obese mice [37].

Similarly, estrogens are thought to contribute to the etiology of intrahepatic cholestasis during pregnancy, which is associated with an increase in the total bile acid pool [38]. This disease usually develops in the third trimester of pregnancy when concentrations of estrogens are the highest. These patients can develop cholestasis outside pregnancy when they are taking oral contraceptives containing 17αethinyloestradiol. High doses of estradiol and its metabolites also cause cholestasis in rodents, and mice lacking estrogen receptors are resistant to these effects [39, 40].

In this review, we will discuss data indicating that bile acids can regulate steroid homeostasis and interfere with steroid signaling pathways through FXRα. This hypothesis relies on studies demonstrating that FXR α is expressed in many steroidogenic tissues. Moreover, bile acids, FXRα ligands, and steroids are derived from the same precursor molecule (cholesterol). Although several of the reported data rely on in vitro studies, they highlight the need to understand these interactions because they may affect health.

Impact of bile acids on glucocorticoid pathways

Glucocorticoids are produced by the adrenal glands and are essential for life. In humans, cortisol is the most important glucocorticoid (GC). The name of the GCs is based on their well-established roles in glucose metabolism during the stress response. GCs are involved in the stimulation of gluconeogenesis, particularly in the liver, the mobilization of amino acids from extrahepatic tissues, the inhibition of glucose uptake in muscle and adipose tissue, as well as the stimulation of fat breakdown in adipose tissue [41]. GCs regulate or support cardiovascular, metabolic, immunologic, and homeostatic functions. The adrenals also produce mineralocorticoids, mostly aldosterone. The main target of aldosterone is the distal tubule of the kidney, where it stimulates the exchange of sodium and potassium.

The potential role of FXRα in adrenals was expected as it was described to be highly expressed in the adrenocortical cells of the *zona fasciculata* [42, 43]. These potential interactions with GC metabolism could be either at the level of GC synthesis, catabolism, or either through the alteration of their physiological functions.

Glucocorticoid synthesis

As summarized in Fig. 1, the impact of FXR α on GC synthesis was recently demonstrated. *Fxrα*$^{-/-}$ mice show the same plasma glucocorticoid concentrations as wild-type mice, suggesting that FXR α might not be involved in the regulation of the adrenal steroidogenesis under normal conditions. However, FXRα might have an impact in adrenal physiology in mice [44], as its activation increases the expression of scavenger receptor class B, member 1 (SR-BI), which is involved in the transport of cholesterol esters, the speciȧc cellular cholesterol pool used for steroidogenesis [45]. C57BL6 female mice treated with Gw 4064, a synthetic FXRα agonist, have an increased plasma corticosteroid concentrations [46].

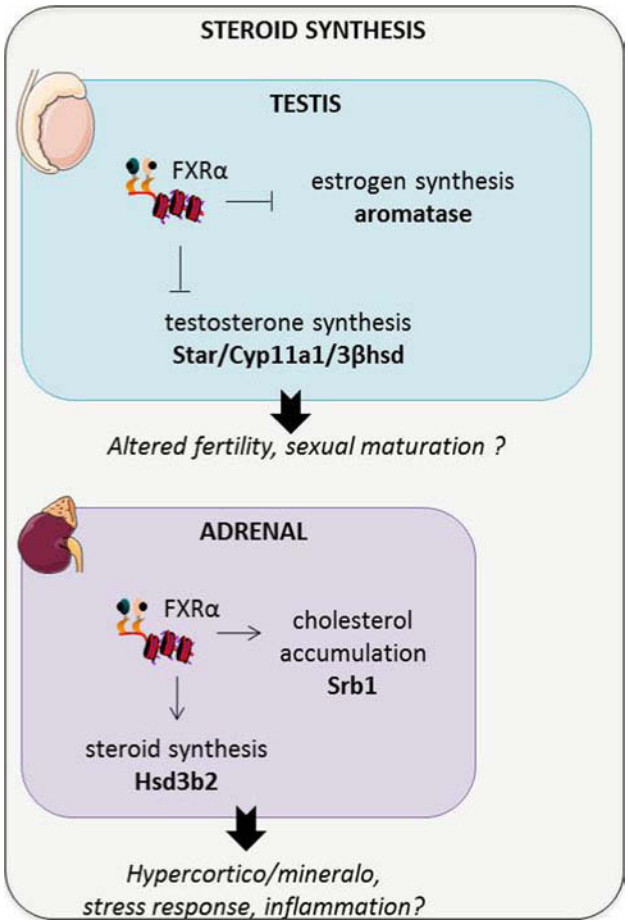

Fig. 1 Schematic representation of FXR α impact on steroid synthesis. In the testes, FXR α regulates the synthesis of both androgens and estrogens through the inhibition of the steroid acute regulatory proteins, cytochrome P450A1, 3 β-hydroxydehydrogenase, and aromatase. This effect can lead to altered male fertility or sexual maturation at puberty. In the adrenal glands, the impact of FXR α on steroid synthesis is mediated through the regulation of the Srb-1 gene, leading to increased cholesterol mobilization for steroid synthesis and regulation of 3 β-hydroxydehydrogenase type 2 in human adrenal cells. This effect can result in hypercorticosteroid or hypermineralocorticoid production and affects stress responses and inAammation

In the human adrenocortical cell line H295R, the use of Gw 4064 and CDCA shows that FXRα positively regulates the expression of 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2). This regulation does not exist in mice, which is consistent with the fact that no FXRe was identiȧed in the mouse Hsd3β1 promoter region, the orthologue of human HSD3B2 gene [47].

Glucorticoid catabolism

In target tissues, the concentration of steroids results from the equilibrium between synthesis and catabolism processes. The eȧciency of GCs relies on an inacti vation or a degradation of the steroids.

Cellular availability The ability of cells to respond to glucocorticoids and aldosterone is dependent on 11β-hydroxysteroid dehydrogenases (11betaHSDs), which catalyze the reversible conversion of physiologically active glucocorticoids to the inacti ve 11-ketometabolites. There are two isoforms of the 11β-hydroxydeshydrogenease: 11β-HSD1 and 11β-HSD2, and 11β-HSD deȧciency is responsible for the hypermineralocorticoid, which results in hypertension.

There is much evidence to suggest that bile acids are able to enhance the intracellular availability of cortisol by abrogating the 11 β-HSD2 activity. BA-dependent inhibition of 11βHSD2 enzyme activity was demonstrated using total renal microsomes. various BAs, such as CDCA and DCA, are able to inhibit the oxidative activity of 11βHSD2. However, in vitro studies suggest that CDCA might affect the activity of 11β-HSD2 in HeK-293 cells only at very high non-physiological concentrations. Consistently, the induction of cirrhosis by bile duct ligation decreased the transcriptional levels of the 11 β-HSD enzyme (Fig. 2). Inhibition of 11βHSD2 may contribute to the sodium retention and potassium excretion observed in patients with liver cirrhosis or cholestasis [48, 49].

In agreement with reports showing that bile acids can inhibit both 11 β-HSD isoforms in various tissues, it was demonstrated that CDCA inhibited 11β-HSD1 in Leydig cells [50]. It can be hypothesized that FXRα can regulate the impact of cortisol in Leydig cells through the regulation of 11β-hydroxysteroid dehydrogenase 1 (Fig. 2). It is well established that glucocorticoids play a critical role in the control of Leydig cell function. High levels of glucocorticoids are associated with a reduced circulating testosterone level and with reproducti ve dysfunction [51, 52]. It has been reported that excess corticosterone reduces the expression and activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17 β-hydroxysteroid dehydrogenase (17β-HSD) in adult rat Leydig cells in vivo and in vitro [53].

Glucocorticoid degradation Glucuronidation is catalyzed by enzymes belonging to the uridine 5, diphosphate glucuronosyl transferase family (UGT) [8, 54]. These enzymes have been divided into two major subfamilies, UGT1A and UGT2B, based on their amino acid sequence homology. The UGT2 family includes enzymes that are able to glucuronidate both bile acids and steroids.

expressed in the liver, kidney, brain, and gastrointestinal tract, *UGT2B7* is considered the major human mineralocorticoid and glucocorticoid metabolizing UGT enzyme (Fig. 2). It has also been demonstrated that a single mutation in this gene greatly affects the level of aldosterone glucuronidation.

Ugt2b7 expression seems to be repressed by LCA treatment in vitro. LCA-FXR α activation dramatically

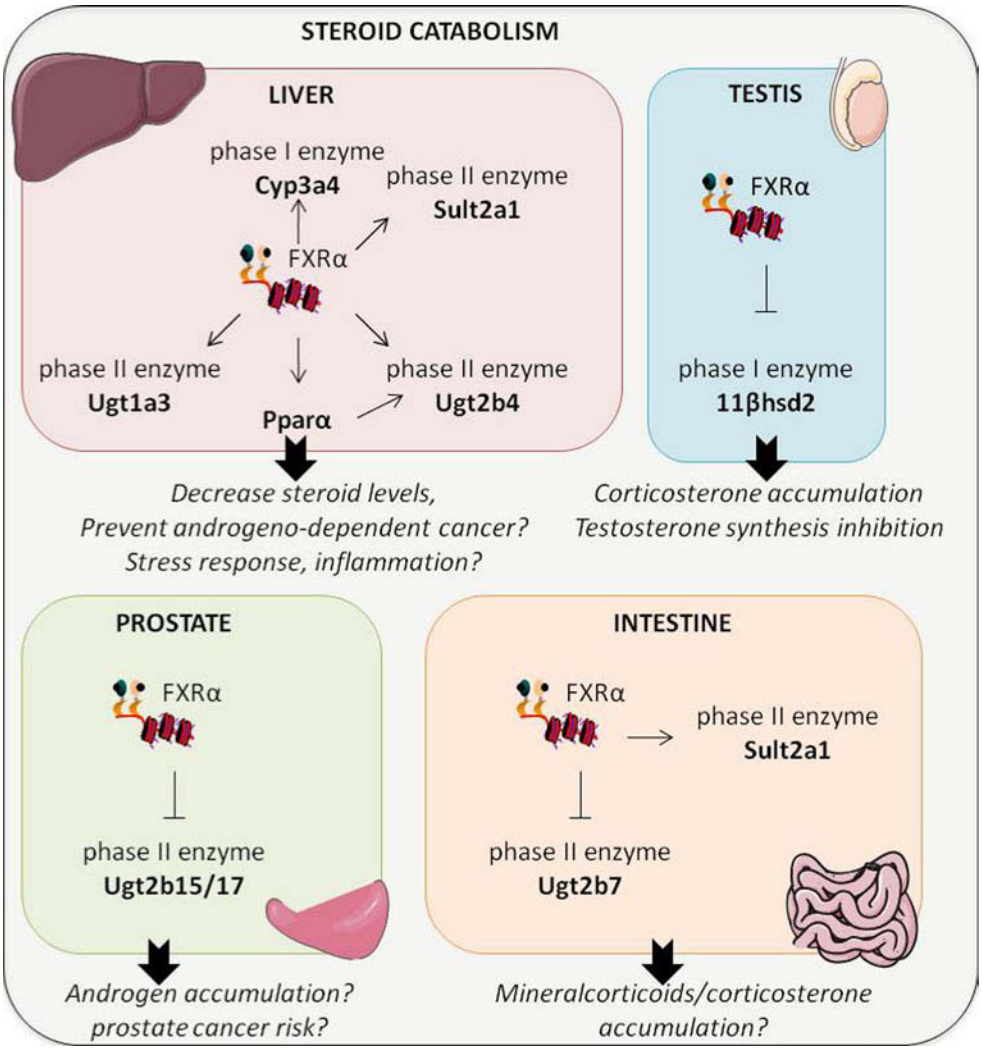

Fig. 2 Schematic representation of FXRα impact on steroid catabolism. In the liver, FXRα participates in the homeostasis of steroids by regulating the expression of many genes involved in steroid metabolism, such as Cyp3a4, Sult2a1, Ugt1a3, and Ugt2b4. Ugt2b4 regulation can be either direct or through PPARα. These types of regulation are also observed in the intestine (Sult2a1 and Ugt2b7). Modulating the local levels of mineralocorticoids and corticosteroids contributes to local inflammation processes. In the testis, the role of FXRα in BA repression of 11βhsd is unclear, but it may contribute to the repression of testosterone synthesis by FXRα. In the prostate, the repression of Ugt2b15/17 expression by FXRα leads to androgen accumulation and an increased risk of prostate cancer development

decreased accumulation of *UGT2B7* mRNA levels through the binding of FXRα to a negative FXRe [55]. Moreover, transfection of cells with hFXRα resulted in a significant suppression of *UGT2B7* expression in the absence of LCA and additional suppression was observed when the cells were treated with LCA.

FXRα interferes with glucose metabolism regulation by glucocorticoid signaling pathways

Regarding the impact of FXRα on GCs synthesis and catabolism, it is reasonable to speculate that bile acids could contribute to a pathologically increased serum level of mineralocorticoids and glucocorticoids [56].

The action of glucocorticoids is mediated through the glucocorticoid receptor (GR), which is a member of the nuclear receptor superfamily that regulates numerous transcription programs including immune suppression, anti-inflammatory responses, and glucose metabolism. Glucocorticoid binds to GR in the cytoplasm and promotes its translocation to the nucleus. Then, activated GR binds to a GR response element (GRe) in the promoter of downstream target genes and allows their transcription through the recruitment of various co-activators such as PGC-1.

GR is highly expressed in the liver, where it regulates the expression of rate-limiting enzymes in gluconeogenesis and plays an important role in the control of glucose metabolism. During conditions of high energy demand, systemic glucocorticoid concentrations increase and activate GR in the liver, leading to glucose mobilization via the expression of gluconeogenesis enzymes [57]. Among these enzymes, phospho(enol)pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) are known to be positively regulated by glucocorticoids and also by glucagon, which both have strong gluconeogenic actions, while insulin suppresses hepatic gluconeogenesis [58]. Conditional mice harboring a disrupted GR in hepatocytes exhibit profound hypoglycemia after prolonged food withdrawal and

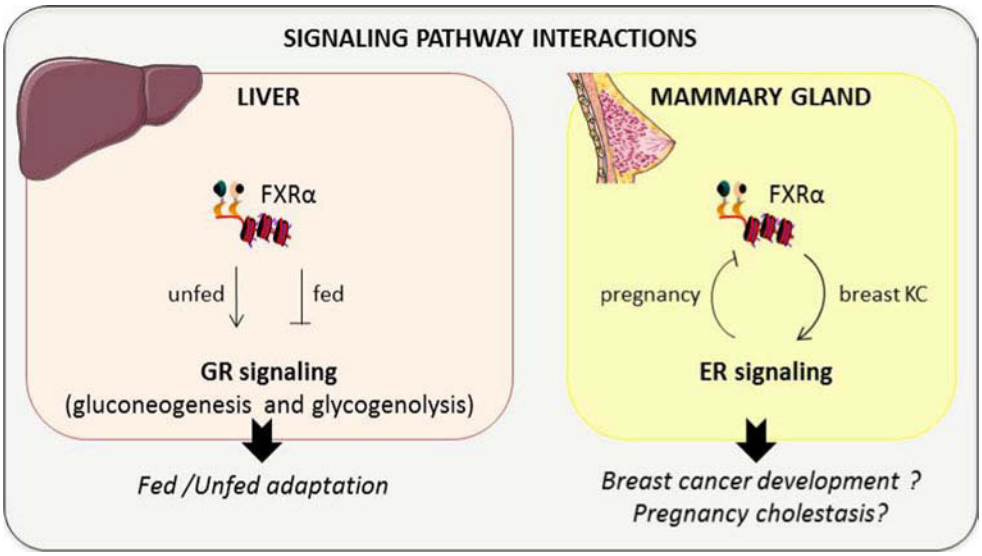

Fig. 3 Schematic representation of the crosstalk between steroids and FXRα signaling pathways. In the liver, the interaction of FXR α and glucocorticoid pathways is notably complex and can lead to either the induction or repression of this pathway. This process is correlated with fed/unfed adaptation processes. FXRα has a role in breast cancer. The expression of FXRα was significantly correlated with proliferation in post-menopausal women with lower estrogen concentrations who had ER-positive breast tumors. Additionally, estrogen levels are thought to contribute to the etiology of intrahepatic cholestasis in pregnancy

are unable to up-regulate the expression of gluconeogenic enzymes [59].

Bile acids exert regulatory effects to maintain glucose and insulin homeostasis. However, the precise role of FXRα in the regulation of hepatic glucose metabolism remains controversial, as it was described either as an inhibitor or an inducer of gluconeogenesis [22, 60, 61] (Fig. 3).

FXRα represses gluconeogenesis Several studies report a repressive effect of FXRα on the expression of gluconeogenic genes, suggesting that FXRα signaling could interfere with/counteract the role of glucocorticoids and GR signaling on hepatic glucose metabolism. C57BL6 mice treated with a 1 % CA-supplemented diet for 7–8 days showed decreased hepatic *Pepck* and *G6Pase* mRNA levels [62, 63]. The involvement of FXRα in *Pepck* and *G6Pase* downregulation was suggested in vivo by Zhang et al. [64], showing that oral Gw 4064 treatment, as well as adenoviral-mediated hepatic overexpression of FXRα, improved hyperglycemia in db/db diabetic mice. Feeding a CA-enriched diet has been shown to decrease fasting blood glucose levels associated with reduced expression of *Pepck* and *G6Pase* mRNA in wild-type but not $Fxr\alpha^{-/-}$ mice. This effect was shown to be mediated through the classical FXRα target gene *Shp*, a known regulator of gluconeogenesis [65].

FXRα induces gluconeogenesis Some in vivo studies showed that treatment with the FXR α agonist Gw 4064 induces *Pepck* mRNA levels in an FXR α-dependent manner [35, 64]. Renga et al. [66] demonstrated that FXR α activates gluconeogenic pathways in the liver through the direct regulation of GR expression and activity. In *Fxr*-null mice, the decreased accumulation of rate-limiting gluconeogenic enzymes after a period of 15 h of withdrawal is associated with blunted liver expression of GR. Treating wild-type mice with a semisynthetic FXR ligand (6e-CDCA) increases the liver expression of GR, *Pepck*, and *G6pase* mRNA accumulation. $Fxr\alpha^{-/-}$ mice failed to regulate *Pepck* and *G6Pase* in response to dexamethasone, suggesting that FXRα is essential for mediating GR gluconeogenic signaling. GR silencing by siRNA in vitro or its pharmacological antagonism in vivo with mifepristone reverses the effect of FXRα activation on the expression of gluconeogenic genes, suggesting that an FXRα-GR pathway regulates the activation of hepatic gluconeogenesis in the transition from the unfed to the fed state.

A diet issue? Such complex regulation of glucose metabolism in vivo has been recently addressed by Ma et al. [65] who showed that the activation of FXRα exerts opposite effects during unfed or fed conditions. In fed animals, the activation of FXRα downregulates the expression of *Pepck* and *G6Pase*, and the opposite effect was observed in the unfed state.

$Fxr\alpha^{-/-}$ mice were defective in the induction of gluconeogenic genes, including *Pgc-1α* and *Pepck*, after 6 h of fasting and displayed lower basal hepatic glucose production, leading to an early hypoglycemia response. This defect in the starvation response was associated with a

signiἀcantly reduced hepatic glycogen content in the *Fxrα*$^{-/-}$ mice [67].

At the molecular le vel, such a model may be partially explained by the relative afἀnity of FXRα to different types of response element. It is well known that FXRα binds with high afἀnity to IR-1, while e R-8 sequences have low-afἀnity binding sites for FXR α [68, 69]. In the fed state, when *Fxrα* mRNA levels are low, the activated receptor regulates the transcription of target genes mainly by binding to IR-1 sequences such as the *Shp* target gene, a kno wn repressor of gluconeogenesis. w hen *Fxrα* mRNA levels are high, as in the unfed state, the receptor might occupy low-afἀnity binding sites, such as the e R-8 sequences, which then allow induction of *Gr* transcription by FXR α. This induction of GR leads to increased *Pepck* and *G6pase* mRNA levels.

Integrative conclusions

Together, these data suggest that B A might have an impact on glucocorticoid metabolism at multiple levels. The results obtained in vi vo using mice treated with a FXR α agonist correlated with the use of Fxr α$^{-/-}$ mice and demonstrate the induction of glucocorticoid levels (Fig. 1).

The increase of GC concentrations follo wing BA exposure is also a result of the increased bioa vailability of glucocorticoids and is associated with decreased li ver catabolism and reduced inactivation by 11βHSD2 (Fig. 2).

The activation of FXRα by BA interferes with GC action on glucose metabolism in li ver. w hen these data are com bined with the well-established role of GR signaling on glucose metabolism, especially during adaptation between fed/unfed conditions, the data suggest that FXRα modulates the kinetics of glucose homeostasis during f asting. This result highlights a potential positive interaction between the two receptors for metabolic adaptations (Fig. 3).

The links between FXR α and GC on li ver glucose metabolism were analyzed by Ma et al. for crosstalk between receptors. Thus, it is important to consider the ligand bioavailability. According to the h ypothesis by Ma et al., the impact of B A/FXRα on glucose metabolism depends on the transition between the fed and unfed states, which corresponds to unstressed/stress situations. The impact of BAs on metabolism will be difἀcult to assess in normal physiology because during f asting, the GC le vels are increased, and B A levels are decreased. The relevance of BAs on GR signaling pathw ays is difἀcult to inte grate. However, it is important to e xamine these parameters in diseases with high BA levels.

Impact of bile acids on sex hormone pathways

There are limited data available on the interactions between bile acid/FXRα signaling pathw ays and se x steroid metabolism. As for GCs, these interactions will be at the levels of synthesis, catabolism, or by modiἀcation of the physiological functions of sex steroids.

Sex hormone synthesis

The only link between FXR α and se x hormone synthesis thus far has been obtained from studies on the testes. The testes are composed of seminiferous tub ules outlined by a basal membrane that separates them from the interstitial compartment. In the adult, these tw o compartments ensure the exocrine (spermatozoa production) and the endocrine (hormone synthesis) functions [70]. The testes are involved in the synthesis of estrogens and testosterone. In males, androgens are responsible for the maintenance of fertility and the de velopment of secondary se xual characters [71]. In addition, testosterone is essential for reproductive function, muscle and bone mass maintenance, cogniti ve function and other ph ysiological parameters. Altered testicular functions may increase the risk of metabolic syndrome [72]. The testes also produce estrogen, which acts through the estrogen receptors e Rα and e Rβ. e strogen down-regulates the luteinizing hormone receptor (Lhcgr) and inhibits the Steroidogenic acute regulatory protein (StAR) gene and others genes implicated in steroidogenesis in Leydig cells [73].

In 2007, v olle et al. [23] detected transcripts of *Fxrα* in the interstitial cells. FXRα was also described as a re gulator of the aromatase gene in tumor Le ydig cells [24]. A decrease in aromatase expression was observed after induction with G w 4064 or CDCA in Le ydig cell lines in vitro (Fig. 1). FXRα interferes ne gatively with SF1 acti vity on sequences of the PII promoter of aromatase, where FXRα enters in competition with SF-1 for binding a common site. This results in an inhibition of estrogen synthesis.

The in vi vo role of FXR α on androgen synthesis w as demonstrated using a FXR α synthetic agonist (G w 4064). If testosterone concentrations were similar between wild-type and *Fxrα*$^{-/-}$ mice, the administration of Gw 4064 repressed steroidogenesis after 12 h of treatment and w as associated with a decrease in *Star*, *Cyp11a1*, and *3β-hsd* gene expression (Fig. 1). At the molecular le vel, it w as demonstrated that this repressive effect is consistent with the up-regulation of *Shp*, which then inhibits LRH-1 and SF1 activity, two know inducers of steroidogenesis. It has also been shown that FXRα regulates the synthesis of androsterone [74].

Bile acids and sex hormone catabolism

The potential interaction of FXR α with se x hormone metabolism was also demonstrated through the re gulation of the catabolism of these steroids. Such cross-talks occur

via the regulation of several key genes as highlighted in Fig. 2 and detailed below.

CYP3A4 expressed predominantly in the adult liver and intestine, the cytochrome P450 enzyme CYP3A4 has been shown both in vitro and in vivo to hydroxylate BAs at the 1β, 6α, and 6β positions, thus participating in the elimination of BAs [75, 76]. Several studies have shown that bile acids positively regulate *Cyp3a11* expression, the mouse orthologue of *Cyp3A4*, to initiate their catabolism when they are in excess [75, 77–79]. CYP3A11 plays an important role in the metabolism of both exogenous drugs and endogenous compounds such as cortisol [80], testosterone [81] or estradiol-17β [82]. This result suggests a potential association with steroid metabolism.

Studies using the HepG2 cell line exposed to either the natural ligand CDCA or to the speciἀc synthetic ligand Gw 4064 have demonstrated that FXR α controls *Cyp3A4* expression in the liver [79]. This result was also observed in the mouse, as wild-type mice fed Gw 4064 have increased hepatic levels of *Cyp3a11*, which is the rodent homologue of human CYP3A4 [79]. However, this effect was not observed in *Fxrα*$^{-/-}$ mice.

epidemiological and clinical evidence links a *CYP3A4* promoter variant (*CYP3A4***1B* (rs2740574)) with the incidence of prostate cancer and the clinical grade of the tumor and disease progression [83–85]. There is no signiἀcant association between the *CYP3A41B** genotype and the levels of serum testosterone. This result suggests that CYP3A4 might have a minimal impact on hepatic catabolism [86]. The decreased *CYP3A4* expression within prostate tissue is associated with a higher Gleason score and poorer cancer-speciἀc survival [87, 88]. This result suggests that CYP3A4 may play a critical role in maintaining androgen homeostasis within the prostate and loss of CYP3A4 leads to the development of cancer.

SULT2A1 In addition to hydroxylation, sulphate or glucuronidate conjugation are important mechanisms for the detoxiἀcation of steroids. Upon conjugation, the substrates become more polar, less toxic and more water soluble, facilitating their clearance.

Dehydroepiandrosterone-sulphotransferase (SULT2A1/Sult2a1) are phase II metabolizing enzymes that catalyze the sulphating of various exogenous chemicals and endogenous compounds including testosterone, estrogen, and BAs [89–91].

Increased serum and urine levels of sulphated BAs were described in patients with cholestatic liver diseases [92, 93]. These clinical observations suggest a potential involvement of BAs in controlling their own sulphating though the regulation of enzymes such as *Sult2a1*. This is consistent with the fact that Sult2A1 is expressed abundantly in the liver and intestine, the two ἀrst-pass metabolic tissues where FXRα is also expressed. Song et al. have demonstrated that primary bile acid CDCA treatment was shown to induce rat/mouse Sult2a1 promoter activity in transfected Caco-2 and HepG2 cells in vitro. This rat/mouse Sult2a1 induction involved an activated FXRα/RXR heterodimer binding to an atypical FXRα response element (IR0) located in the 5′ Ąanking region [90].

However, sequence alignments have shown that this IR0 element does not exist in the human SULT2A1 5′-Ąanking region, demonstrating that the regulation by FXR α might not be conserved between species [94, 95]. Human *SULT2A1* mRNA levels failed to be modulated in cultured human primary hepatocytes treated with CDCA [95]. Further studies are needed to understand how FXR regulates human *SULT2A1* expression.

Consistently, in vivo investigations showed that FXRα may be involved in the repression of basal *Sult2a1* expression. Moreover, CDCA-fed mice presented markedly decreased *Sult2a1* expression in wild-type mice. This is correlated with an increased level of *Shp,* suggesting a FXRα-dependant mechanism, which was conἀrmed by the lack of *Sult2a1* modulation in *Fxrα*$^{-/-}$ mice following a diet [96].

Interestingly, *Fxrα*$^{-/-}$ mice present an increased level of *Sut2a1* in basal physiology and are resistant to LCA induced liver toxicity compared to wild-type (wT) mice [97].

SULT2A1 was originally involved in the inactivation of androgen hormones. In the rodent liver, the high expression of *Sult2a1* during the androgen-insensitive state of the hepatic tissue in senescent males is thought to be a result of the eἀcient inactivation of androgens into androgen sulphates [98, 99]. Thomae et al. [100] reported 3 *SULT2A1* gene alterations that result in decreased *Sult2a1* expression and activity. Interestingly, these alterations were present only in African American patients and were suggested to be partially responsible for the androgen-associated risk of disease. A signiἀcant increase in the DHEA-to-DHEA-sulphate ratio was observed in African American participants with a heterozygous *SULT2A1* A63P/A261T genotype. However, the presence of the different *SULT2A1* alleles was not associated with prostate cancer.

Moreover, as FXR α and AR are both able to control *Sult2a1* gene expression, it is reasonable to think that bile acid and androgen metabolisms could crosstalk through *Sult2a1* regulation [101, 102].

UGTs *Sex steroid catabolism also involves* UDP-glucuronosyltransferases (UGT). Among the 18 functional UGT enzymes identiἀed in humans, UGT2B7, UGT2B15, and UGT2B17 have a remarkable capacity to conjugate androgens [103].

UGT2b7 A tumor-suppressor function was suggested for *UGT2B7* by preventing the accumulation of mutagenesis compounds like 4-hydroxyestrone [104]. Indeed, reduced levels of the UGT2B7 protein and glucuronidation of 4-hydroxyestrone were shown in invasive cancers. However, the role of the bile acid challenge in vivo on UGT2B7 expression still remains to be determined.

UGT2B4 (liver) Barbier et al. [8] identified human UGT2B4 as a target gene of FXRα. Activation of FXRα by CDCA or Gw 4064 in primary human hepatocytes or in the HepG2 cell line resulted in increased *UGT2B4* expression through an atypical binding of FXRα as a monomer to a single hexameric DNA motif.

Interestingly, the PPARα agonist fenofibrate was shown to activate the *UGT2B4* gene promoter through a specific peroxisome proliferator activated receptor (PPAR) response element [105]. DCA also induces the transcription of PPARα gene via an FXRα-mediated mechanism. Thus, bile acids may induce *UGT2B4* expression directly through activation of FXRα and/or indirectly through FXRα-dependent induction of PPARα, which then activates *UGT2B4* transcription. Through these mechanisms, BAs may be part of a negative feedback mechanism by which BAs control their elimination to prevent pathophysiological toxicity.

The potential impact of BAs on steroid metabolism should be taken into consideration as UGT2B4 is known to be active on 5α-reduced androgens and polyhydroxylated estrogens, including estriol, 4-hydroxyestrone and 2-hydroxyestriol.

UGT1A3 (liver) Hepg2 cells treated with LCA, CDCA, and Gw 4064 present increased *UGT1A3* mRNA levels. The resulting CDCA-24-glucuronide was shown to exhibit an antagonistic effect on FXRα as feedback inhibition. *UGT1A3* is expressed in the liver, intestine, and large bowel [106–108]. Apart from BA, UGT1A3 metabolizes xenobiotics such as polyaromatic hydrocarbons as well as estrogens, and vitamin D derivatives [109–111]. Because *UGT1A3* is significantly induced by FXRα in response to bile acids, this regulation could link bile acid metabolism and steroid hormone metabolism alteration.

UGT2B15/17 (prostate) While glucuronidation was generally considered to be a hepatic/intestinal detoxification mechanism, extrahepatic glucuronidation is now established as an efficient way to locally inactivate endogenous bioactive molecules [103, 112]. This is particularly true for androgens, which are efficiently glucuronidated within their target tissues, such as the human prostate [113].

A regulatory function of the nuclear receptor FXRα in androgen metabolism has been shown in prostate cancer LNCaP cells [114]. CDCA or Gw 4064 repress gene expression and androgen-conjugating activity of the UGT2B15 and UGT2B17 enzymes in prostate cancer LnCaP cells. The regulation of UGT2B15 expression by FXRα seemed to be tissue-specific, as previous data have shown that CDCA does not modulate *UGT2B15* mRNA in human hepatocytes [8]. Moreover, *Fxrα*$^{-/-}$ mice present an increased level of *UGT* mRNA accumulation in the prostate compared to wild-type mice. The exact mechanism by which FXRα negatively regulates *UGT2B15* and *UGT2B17* genes, and the physiological implications of this regulation, remain to be determined because androgen glucuronidation is almost absent in the rodent prostate [114–116].

In contrast, the importance of glucuronidation for androgen metabolism in the human prostate was highlighted by the observation that polymorphisms within androgen-glucuronidating genes are associated with an increased risk for prostate cancer [117, 118]. A *UGT2B17* inactivation polymorphism was associated with an increased prostate cancer risk [119]. The D85 polymorphism of UGT2B15, which leads to a less efficient protein for conjugation of 3-diol and DHT, results in higher androgen exposure in prostate tissue. In accordance with these findings, the D85 allele has been reported to increase prostate cancer risk and aggressiveness [120, 121].

During cholestasis, plasma levels of bile acids are drastically increased [122], and it can be hypothesized that in such patients, glucuronidation of androgens may be reduced, resulting in the accumulation of androgens in the prostate, which may correspond to a pro-carcinogenic mechanism. Interestingly, the development of cholestasis has been reported in various patients with prostate cancer [123–125].

This conclusion is particularly important because FXRα agonists are currently considered as a promising treatment of several diseases such as hepatitis C or metabolic syndrome, as highlighted by several ongoing clinical trials (www.clinicaltrials.org).

FXRα interferes with sex hormone signaling pathways

Potential impact of FXRα on AR pathways (Fig. 3) The role of FXRα on androgen signaling pathways has not been clearly demonstrated and is still speculative. However, it can be hypothesized that by controlling *Shp* expression, FXRα could interfere with the actions of androgen. SHP is able to interact with numerous nuclear receptors. It has been demonstrated in vitro using GST -pull-down experiments that SHP interacts and inhibits the androgen receptor activity [126]. SHP acts by competing with AR co-activators. These data opened a new field of research concerning how BAs might interfere with androgen signaling pathways. This is

even more interesting in line with the described inhibitory effect of FXRα synthetic ligand on testosterone production.

Potential impact of FXRα on ER Any impact on breast cancer? (Fig. 3). Although no data describe links between FXRα and female hormone synthesis, reports suggest a potential role for FXRα in breast pathophysiology. e strogen exposure has long been known to contrib ute to the etiology of breast cancers [127], and approximately tw o-thirds of these cases are characterized by dysre gulation of the estrogen receptor α (e Rα) signaling [128]. Therapy consists of blocking estrogen synthesis (aromatase inhibitors) or e R transactivation (estrogen receptor modulators, such as tamoxifen) [129, 130]. e vidence suggests a potential role for bile acids in breast cancer etiology (Fig. 3). The accumulation of bile acids has been reported in breast c yst Ąuid and has been proposed as a potential risk f actor for breast cancer [131–133]. w omen with breast cancer may ha ve differences in the fecal e xcretion of BAs compared to controls [134–136]. In addition, long-term follow-up of women undergoing cholecystectomy has re vealed a higher risk of breast cancer [137].

The potential in volvement of FXR α was highlighted in several clinical studies. Interestingly, FXRα was shown to be expressed in normal breast tissue, and se veral studies established signiȧcant correlations between FXR α and e R expression in breast cancer samples [138, 139]. Fifty percent of e R-negative breast cancer samples had weak FXR α expression, and 70 % of e R-positive samples had FXR α expression suggestive of crosstalk between e R and FXR α signaling [139]. These clinical data are supported by semi-quantitative analyses re vealing that the e R-positive breast cancer cell line MCF-7 has higher FXR α protein accumulation than the e R-negative MDA-MD 231 cell line [37]. A signiȧcant correlation between FXR α and the Ki67 proliferative marker has also been observ ed. FXRα expression was signiȧcantly correlated with proliferation in patients with e R-positive breast tumors in postmenopausal w omen, with lower estrogen concentrations [37]. In the conte xt of low estrogen, FXR α expression may play a k ey role in proliferation. This hypothesis is further supported by the presence of high plasma levels of DCA in postmenopausal breast cancer patients [140], suggesting that bile acids might be in volved in the onset and de velopment of mammary gland cancers in an estrogen-independent conte xt through the activation of FXRα.

If these correlations are established in patients, the molecular mechanisms remain unclear with contradictory results.

In vitro data sho w that activated FXRα induces a mitogenic response in a breast cancer cell line through posi tive crosstalk with the e R. Indeed, the e R-positive cell line MCF-7 sho ws increased proliferation in response to FXRα activation. This was associated with a pro-estrogenic response, as measured by the do wnregulation of e Rα accumulation [37, 139]. These results suggest that in absence of estrogens, these pro-estrogenic patterns should a result of FXR α-mediated activation of e Rα dependent transcription. At a relati vely low concentration, CDCA glucuronidate increased MCF-7 gro wth combined with this same pro-estrogenic ef fect (decreased e rα accumulation and up-re gulated e R target genes). Ho wever, while low doses of CDCA lead to this same estrogenic response, a contradictory decrease in proliferation w as observed. These discrepancies might be explained by dose effects of bile acids on proliferative pathways through e R activation [141].

In contrast to pre vious reports [37, 139], Giordano et al. show that CDCA or Gw 4064 inhibited proliferation in the breast cancer cell line MCF-7 and in the Tam-resistant breast cancer cell line MCF-7/TR1. This anti-proliferative effect of FXR α suggests repression of the H e R2 receptor, perhaps by enhancing formation of a FXR α and NF-κB complex inhibiting the binding of NF- κB to its responsive element located in the human H e R2 promoter region.

High concentrations of FXRα ligands exert an anti-proliferative effect on breast carcinoma cell lines, regardless of their e R status. In breast cancer cell lines, FXR α agonists down-regulated the breast cancer tar get gene aromatase. These data could also be rele vant, as aromatase inhibition is classically used in breast cancer treatment.

Integrative conclusion

Taken together, these data demonstrate complex interactions between BAs and sex hormone homeostasis. Several ȧndings suggest that B As decrease sex hormone synthesis (testosterone and estrogen) in male mice (Fig. 1), but this result has not been demonstrated in female mice and or in humans. The impact of the B As/FXRα pathways in lowering the le vels of se x hormones is also dependent on their effects on li ver catabolism through the re gulation of genes such as *Sult2a1* and *Cyp3a* (Fig. 2). These data are consistent with the kno wn decrease in plasma testosterone le vels in a male experimental model of liver injuries [142].

A decrease in sex hormone levels in males may have major effects on sexual maturation and/or the maintenance of secondary sexual characters.

Hormone concentrations are ȧnely controlled in tar get organs such as the prostate. In cell lines, there is e vidence for a link between FXR-mediated maintenance of B A homeostasis and hormone steroid inacti vation. It has been hypothesized that FXR α transactivation in li ver or pros tate tissues may prevent androgen accumulation and the development of androgen-dependent cancers such prostate

cancer through the regulation of Ugt2b15/17. However, there are no data describing FXRα dependent regulation of Cyp3A4 within the prostate.

The interaction of FXRα with the estrogen receptor in breast cancer (Fig. 3) supports crosstalk between FXRα and eR in inducing tumor progression. However, no clear in vitro evidence has been provided. This issue highlights the need for a better understanding of these pathways to determine if FXRα agonists/antagonists could be useful drugs in some cases of breast cancers.

Conclusions and perspectives

BAs represent the main cholesterol catabolites. Because they share the same origin as steroids, there may be some crosstalk between BA metabolism and steroids. Recent studies have focused on bile acids and their nuclear receptor, FXRα (NR1H4).

Glucocorticoids and estrogen can inhibit BA-FXRα signaling pathways in the liver and mammary glands. Alternatively, as reported in this review, FXRα/BA pathways can affect steroid metabolism at the levels of synthesis, catabolism and downstream signaling pathways. This is highlighted by the fact that FXRα is expressed in steroidogenic tissues, such as the adrenal glands and the testis, where FXRα controls steroid production. FXRα also interferes with steroid signaling pathways in target tissues such as the liver through crosstalk with the glucocorticoid receptor (GR). The evidence for crosstalk between the FXRα/BA pathways and steroids has been reinforced by the recent identiἀcation of the impact of glucocorticoids and estrogen on the activity of FXRα.

Several FXRα polymorphisms have been detected in humans, and they are associated with pathologies including obesity and gallstone diseases, such as cholelithiasis or intrahepatic cholestasis of pregnancy (ICP) [143, 144], [145, 146]. The role of FXRα in the metabolism of either glucocorticoids or sex hormones could be used to determine if altered FXRα signaling pathways are involved in idiopathic diseases. Screening patients for FXRα polymorphisms could offer new insight into the origins of these pathologies and determine if FXRα is a diagnostic/prognostic marker.

The crosstalk between BAs and steroid metabolism has important roles in health. Further studies are needed to clearly identify all of the pathways activated by BAs through FXRα. The complexity of the system is important to understand because the activation of this receptor could lead to both beneἀcial [147] and deleterious effects. This phenomenon is of particular interest when these endogenous molecules abnormally accumulate in pathophysiological conditions, such as liver injury.

The incidence of liver disease is difἀcult to establish because the concept encompasses many different types of pathologies. The onset of many types of liver disease is insidious and is not detected until hepatic decompensation occurs. The real signiἀcance of steroid metabolism in the physiology of liver diseases must be underestimated.

Clinical data have suggested a link between liver dysfunction and male fertility disorders [148, 149]. Experimental models of cholestasis induced by bile duct ligation to increase plasma bile acid levels [150] have been associated with testicular alterations [151, 142]. In this pathological context, reduced plasma testosterone levels have been associated with loss of the germ line in the seminiferous tubules. This effect can result in reduced fertility or infertility and altered male sexual maturation.

The role BA in steroid metabolism should be considered with the fact that FXRα is targeted for pharmacological drugs in the treatment of such diseases as diabetes [152]. It will not be simple to target one BA receptor to manage a speciἀc pathology. Therefore, we will have to consider that interfering with steroid metabolism might lead to deleterious side effects from this therapy.

In addition to their involvement in the control of gluconeogenesis in the liver [41], GCs also regulate or support a variety of important cardiovascular, metabolic, immunological, and homeostatic functions. Therefore, before using FXRα agonists as a long-term treatment for diseases, it will be necessary to ensure there are no deleterious effects on cardiac function, hypertension or immunity.

Studies will be required to determine whether long-term treatment with FXRα modulators affects testosterone synthesis in men and to verify the interactions with AR signaling pathways. The inhibition of androgens could affect secondary sexual characteristics. Additionally, there could be a long-term impact on pathologies, including the development of prostate cancer.

Pregnancy cholestasis leads to an increased risk of preterm delivery and perinatal mortality [153]. This condition is associated with increased bile acid levels and are treated with either cholestyramine or ursodeoxycholic acid. Previous reports have described a role for FXRα during pregnancy, and it is associated with increased hepatic bile acid concentrations in mice and reduced FXRα function [154]. Thus, we must analyze mouse models to determine the impact of FXRα agonists on fetal health before treating patients with preclinical diabetes.

Perspectives

As FXRα agonists are explored as potential therapeutic drugs for the treatment of several diseases, the development of strategy to avoid systemic effects will be needed. It will be necessary to target this receptor in a cell-speciἀc

manner. It will be important to take into consideration the patient history (hormonal status) for developing personalized therapy. This highlights the fact that drug development must rely on a strong fundamental research in integrative physiology and that drugs cannot be developed based on molecular mechanisms characterized in vitro or in vivo in a single organism. This is a challenging, open field for future research that will require interdisciplinary approaches.

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Bile Acids Alter Male Fertility Through G-Protein-Coupled Bile Acid Receptor 1 Signaling Pathways in Mice

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Bile acids (BAs) are signaling molecules that are involved in many physiological functions, such as glucose and energy metabolism. These effects are mediated through activation of the nuclear and membrane receptors, farnesoid X receptor (FXR-α) and TGR5 (G-protein-coupled bile acid receptor 1; GPBAR1). Although both receptors are expressed within the testes, the potential effect of BAs on testis physiology and male fertility has not been explored thus far. Here, we demonstrate that mice fed a diet supplemented with cholic acid have reduced fertility subsequent to testicular defects. Initially, germ cell sloughing and rupture of the blood-testis barrier occur and are correlated with decreased protein accumulation of connexin-43 (Cx43) and N-cadherin, whereas at later stages, apoptosis of spermatids is observed. These abnormalities are associated with increased intratesticular BA levels in general and deoxycholic acid, a TGR5 agonist, in particular. We demonstrate here that *Tgr5* is expressed within the germ cell lineage, where it represses *Cx43* expression through regulation of the transcriptional repressor, T-box transcription factor 2 gene. Consistent with this finding, mice deficient for *Tgr5* are protected against the deleterious testicular effects of BA exposure. *Conclusions:* These data identify the testis as a new target of BAs and emphasize TGR5 as a critical element in testicular pathophysiology. This work may open new perspectives on the potential effect of BAs on testis physiology during liver dysfunction. (HEPATOLOGY 2014;60:1054-1065)

The incidence of infertility is constantly increasing and affects up to 25% of couples. Approximately 50% of the cases comprise disorders of the male reproductive system. They have frequently been associated with an inadequate number and quality of male germ cells (e.g., spermatozoa).[1,2] Alterations of testicular physiology play an important role in reduced sperm number, leading to infertility. The major functions of the testes include synthesis of the male sex hormone (testosterone) and production of gametes. Spermatogenesis takes place within the seminiferous tubules in association with the Sertoli cells (SCs), which provide structural support for developing germ cells through cell-cell interactions involving proteins such as

Abbreviations: Abs, antibodies; ALT, alanine aminotransferase; BA, bile acid; BTB, blood-testicular barrier; CA, cholic acid; cDNA, complementary DNA; Cx43, connexin-43; Cyp3a11, cytochrome P450, family 3, subfamily a, polypeptide 11; DCA, deoxycholic acid; DHT, dihydrotestosterone; FXR-α, farnesol X receptor; GPBAR1, G-protein-coupled bile acid receptor 1; H&E, hematoxylin and eosin; IHC, immunohistochemistry; Igf1, insulin-like growth factor 1; IP, intraperitoneal; IT, intratesticular; LC, liquid chromatography; LCA, lithocholic acid; MC, mass spectrometry; mRNA, messenger RNA; OA, oleanolic acid; PFA, paraformaldehyde; SCs, Sertoli cells; siRNA small interfering RNA; Sult2a1, sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1; Tbx2, T-box transcription factor 2; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; Ugt2B34, UDP glucuronosyltransferase 2 family, polypeptide B34; WT, wild type.

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N-cadherin, connexin-43 (Cx43), or E-cadherin.[3] In a testis, SCs form a blood-testicular barrier (BTB), which is essential for optimal spermatogenesis. Moreover, altered interactions between SCs and germ cells (spermatids) can perturb BTB integrity.[5,6] These cellular interactions allow orientation of germ cells during differentiation. This is crucial because disorientation may induce germ cell apoptosis.[7]

The urgency in identifying factors altering male fertility is emphasized by the fact that idiopathic infertility represents 25% of cases.[8] Recently, it was demonstrated *in vivo* that testicular testosterone synthesis was repressed by a synthetic agonist of the nuclear bile acid (BA) receptor, farnesoid X receptor alpha (FXR-α; NR1H4).[9] This suggests that BA signaling could play a role in testicular pathophysiology. In addition to their role in promoting digestion, BAs have also been defined as endocrine factors whose actions are mediated by two BA responsive receptors: the nuclear receptor, FXR-α,[10] and the G-protein-coupled receptor, TGR5 (G-protein-coupled BA receptor; GPBAR1).[11] These receptors are currently considered as potential therapeutic targets[12,13] because they have been demonstrated to be involved in many physiological functions, such as lipid and carbohydrate metabolism[14] as well as energy expenditure.[15,16] Although FXR-α and TGR5 have been reported to be expressed within the testes,[9,17] the effect of BA signaling on testis physiology and male fertility remains elusive.

To define the potential effect of BAs on testicular physiology and fertility, we exposed adult male mice to dietary BA supplementation. We demonstrate that mice fed a diet supplemented with cholic acid (CA) have altered fertility subsequent to testicular defects and lower sperm count. Elevated plasma BA levels led to germ cell sloughing and BTB rupture, as well as apoptosis of spermatids. The use of *Tgr5* null ($Tgr5^{-/-}$) mice highlights that the BA-TGR5 pathway plays a critical role in mediating fertility disorders, some of which are mediated within the germ cell lineage.

Materials and Methods

Ethics Statement. This study was conducted in accord with the current regulations and standards approved by the animal care committee (CEMEA Auvergne; protocol CE 07-12).

Animals. C57Bl/6J mice were purchased from Charles River Laboratories (L'Arbresle, France), and the $Tgr5^{-/-}$ mice used have been previously described.[18] The mice used in this study were maintained in a C57BL/6J background and housed in temperature-controlled rooms with 12-hour light/dark cycles. Mice had *ad libitum* access to food and water. Nine-week-old mice were exposed to a D04 diet (control) or D04 diet supplemented with 0.5% CA (CA diet; SAFE, Augy, France) for 0.5, 1, 2, or 4 months.

Histology. After diet exposure, testes were collected, paraformaldehyde (PFA)-fixed and embedded in paraffin, and 5-μm-thick sections were prepared and stained with hematoxylin and eosin (H&E).

For analysis of BTB integrity, 15 μL of EZ-Link Sulfo-NHS-LC-Biotin (7.5 mg/mL; Thermo Fisher Scientific, Brebières, France) were injected into the left testis of anesthetized males exposed to a control or CA diet.[19] Then, after 20 minutes, testes were removed, PFA-fixed and embedded in paraffin, and 5-μm-thick sections were prepared and stained for biotin.

For determination of cellular localization of bile acids in the testis, we injected 0.6 mg of cholyl-lysyl-fluorescein (BD Biosciences, Le Pont de Claix, France) either in an intraperitoneal (IP; 200 μL) or intratesticular (IT; 15 μL) manner. Thirty minutes after injection, testes were harvested, PFA-fixed and embedded in paraffin, and 5-μm-thick sections were prepared.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Analysis. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) experiments were performed as previously described on 5 μm of testis fixed in 4% PFA.[20] In each testis, at least 100 random seminiferous tubules were counted. Results are expressed as the number of tubules with either spermatocytes or spermatids TUNEL positive per 100 seminiferous tubules.

Immunohistochemistry. Paraffin sections of PFA-fixed testis were sectioned at 5 μm. Sections were mounted on positively charged glass slides (Superfrost plus; Thermo Scientific), deparaffinized, rehydrated, treated for 20 minutes at 93-98°C in citric buffer (0.01 M, pH 6), rinsed in osmosed water (2 × 5 minutes),

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and washed (2 × 5 minutes) in Tris-buffered saline. Immunohistochemistry (IHC) was conducted according to the manufacturer's recommendations, as described earlier.[21] Slides were then counterstained with Hoestch medium (1 mg/mL). Antibodies (Abs) used are given in the Supporting Information (Supporting Table 1).

Endocrine Investigations. Steroids were extracted from testes as previously described.[20] IT and plasma steroid levels were measured using commercial kits: testosterone and estradiol (Diagnostic Biochem, London, Ontario, Canada), dihydrotestosterone (DHT), 3α-androstanediol (MyBioSource, San Diego, CA), and androsterone (Antibody-on-line GmbH, Paris, France).

BA Measurements. Plasma BAs were analyzed in acetonitrile-deproteinized samples by liquid chromatography (LC) tandem mass spectrometry (MS/MS) using a method described previously, with minor modifications.[22] Quantitative measurement of tissue BAs was performed as described before.[23] The qualitative measurement of the BAs in testis extracts was performed by ultra-pressure LC-MS. The sample was injected into a C_{18} guard column (20 × 2 mm) and washed with water; subsequently, BAs and BA conjugates were eluted with acetonitrile in a single peak. During elution of this peak, spectra were taken. For experiments on wild-type (WT) and *Tgr5*$^{-/-}$ mice, BA measurements were performed using enzyme-linked immunosorbent assays, following the manufacturer's recommendations (catalog no.: 80470; Crystal Chem, Inc., Downers Grove, IL).

Real-Time Reverse-Transcriptase Polymerase Chain Reaction. RNA from testis samples was isolated using Nucleospin RNA (Macherey-Nagel, Hoerdt, France). Complementary DNA (cDNA) were synthesized from total RNA with the Moloney murine leukemia virus reverse transcriptase and random hexamer primers (Promega, Charbonnière Les Bains, France). Real-time polymerase chain reaction measurement of individual cDNAs was performed using SYBR Green dye (Master Mix Plus for SYBR Assay; Eurogentec, Angers, France) to measure duplex DNA formation with the Eppendorf RealPlex system. The sequences of primers are reported in Supporting Table 2. Standard curves were generated with pools of testis cDNA from animals with different genotypes and/or treatments. Results were analyzed using the ΔΔct method.

Western Blotting. Proteins were extracted from tissues using lysis buffer (0.4 M of NaCl, 20 mM of Hepes, 1.5 mM of $MgCl_2$, 0.2 mM of ethylenediaminetetraacetic acid, 0.1% NP40, and 1× protease inhibitors; Roche Diagnostics, Meylan, France). Abs were suspended in Tri-buffered saline, 0.1% Tween, and 10% milk. The Abs used are given in the Supporting Information (Supporting Table 1).

Cell Studies. GC1-spg cells were used as previously described.[9] Cells were treated for 24 hours with vehicle (ethanol, 1/1,000), deoxycholic acid (DCA; 5-100 μM; Sigma-Aldrich, St. Louis, MO), lithocholic acid (LCA; 5-20 μM; Sigma-Aldrich), and oleanolic acid (OA; 2-10 μM; Interchim, Montluçon, France). Then, cells were harvested 24 hours later, and messenger RNA (mRNA) or protein extractions were performed.

Transient Transfection. GC1-spg cells were transfected with small interfering RNA (siRNA) using interferin (Ozyme, Saint Quentin Yvelines, France) in six-well plates (100,000 cells per well). The siRNA directed against *Tgr5* or T-box transcription factor 2 gene (*Tbx2*), as well as control siRNA (siGfp), was transfected at 5 ng per well. When 48 hours after the transfection had passed, cells were treated with vehicle (ethanol, 1/1,000) or DCA. Then, cells were harvested 24 hours later, and mRNA extractions were performed.

Cellular Localization. To study *Tgr5* cellular localization within the testis, we used a classical transitory germ-cell loss protocol with busulfan injections.[24] C57BL/6J mice were injected once with 20 mg/kg of busulfan (Sigma-Aldrich). Mice were killed on the day of injection (T0), 4 weeks, or 8 weeks after treatment. For details, see Materials and Methods section in the Supporting Information. We also used spermatocytes, which were isolated and purified as previously described.[20]

Statistical Analysis. For statistical analysis, 2-way ANOVA was performed using the statistical software package SigmaStat 3.0. When significant effects of treatment or genotype or their interactions were obtained, multiple comparisons were made with Holm-Sidak method. All numerical data are mean ± SEM. A P value less than 0.05 was considered significant.

Results

Dietary BA Supplementation Alters Male Reproductive Function. To define the potential effect of BAs on testicular physiology and fertility, we exposed adult male mice to dietary CA supplementation (0.5%) for 4 months. A CA diet led to altered fertility, with 24% of the exposed males unable to produce progeny (Fig. 1A). In males producing progeny, a CA diet also decreased (20%) the number of pups per litter after 4 months of diet (Fig. 1A). This decrease was negatively correlated with the increase in plasma BA

levels ($r^2 = -0.890$; $P = 0.000565$; Fig. 1B). As expected, CA diet increased plasma BA levels (Supporting Fig. 1A). Although CA was the most abundant BA species, DCA and its derivatives were the most highly induced after the CA diet (Supporting Fig. 1B). The decrease of number of pups per litter in CA-diet–fed males was correlated with DCA levels ($r^2 = -0.895$; $P = 0.000468$; Fig. 1B). The reduced fertility was also associated with a lower production of spermatozoa by the testis (Fig. 1C).

Dietary BA Supplementation Alters Germ Cell Survival. A marked increase in germ cell apoptosis, specifically spermatids, was observed after 4 months of CA diet (Fig. 2A,B) and was associated with an induction of cleaved caspase-3 (Fig. 2C). No modification of IT testosterone or other male hormones, such as DHT, androsterone, and 3α-androstanediol, was observed (Fig. 2D and Supporting Fig. 1C). Moreover, gene expression levels of several androgen-dependent genes (*Pem* and *Osp1*) were not changed after 4 months of CA diet (Fig. 2E). These results suggest that androgen-independent mechanisms may underlie apoptotic events in spermatids of male mice exposed to BAs. Interestingly, IHC analysis indicated abnormal polarization of elongating/elongated spermatids in mice exposed to BAs, compared to the control group (Fig. 2F,G).

Integrity of the Seminiferous Epithelium Is Altered Upon Chronic BA Exposure. Histological analyses revealed the presence of cell aggregates in the center of the seminiferous tubules (Fig. 3A,B) as well as destructured tubules (Fig. 3C,D) in testes of the CA-treated group. Whereas cell aggregates were already observed after 2 months of diet, the presence of destructured tubes only appeared after 4 months (Fig. 3B,D). This suggests that cell-cell interaction mechanisms could have been altered by the CA diet, resulting in germ cell detachment.

Use of a biotin-coupled tracer indicated that, after 2 months of diet, the BTB is no longer intact (Fig. 3E,F). Analysis of cell-cell interaction proteins indicated a decreased accumulation of N-cadherin and Cx43 after 2 months of BA exposure (Fig. 3G,H), whereas SRC, claudin-5, β1-integrin, β-catenin, nectin-3, and vimentin were not affected (Supporting Fig. 1D). Furthermore, *Cx43* mRNA accumulation was already altered after 15 days of CA diet exposure, whereas *N-cadherin* mRNA accumulation was unchanged (Supporting Fig. 1E).

TGR5 Deficiency Protects Males From BA-Induced Testis Abnormalities. In contrast to WT males, $Tgr5^{-/-}$ males did not display any effect on male reproductive efficiency (Fig. 4A, left panel). Both the number of pups per litter and the number of spermatozoa in $Tgr5^{-/-}$ males were not reduced in response to 4 months of CA diet (Fig. 4A, right panel, and 4B), indicating that the effects of chronic BA exposure on male reproductive functions are *Tgr5* dependent. Moreover, no morphological abnormalities from chronic CA feeding, such as cell center aggregates or destructured tubules, could be observed in the testis of $Tgr5^{-/-}$ males (Fig. 4C). Consistently, no effect was observed on BTB integrity, apoptosis levels, or

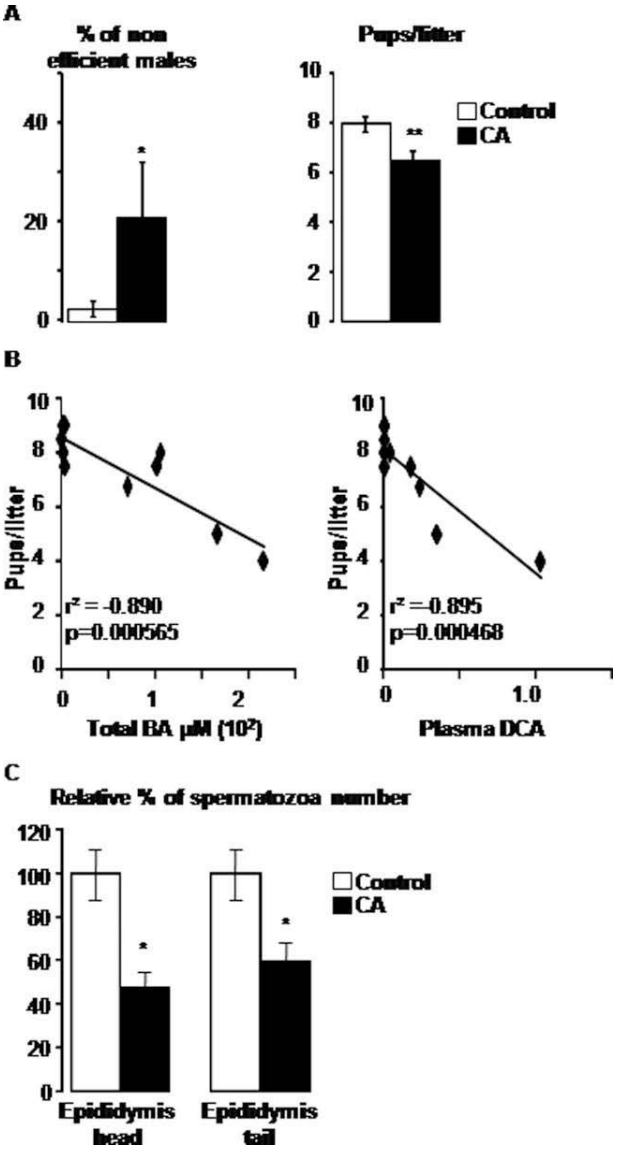

Fig. 1. CA diet induces male hypofertility. (A) Each male was bred with 2 C57Bl/6J females to analyze their capacity to mate (percentage of nonefficient males; left panel) and number of pups per litter obtained (right panel). (B) Correlation of the number of pups per litter with the concentration of total plasma BAs and DCA. (C) Spermatozoa count in the heads and tails of the epidydimis of males exposed to 4 months of control or CA diets (n = 10-20 per group). Control diet group was arbitrarily fixed at 100%. In all of the panels, data are expressed as the means ± standard error of the mean. Statistical analysis: $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

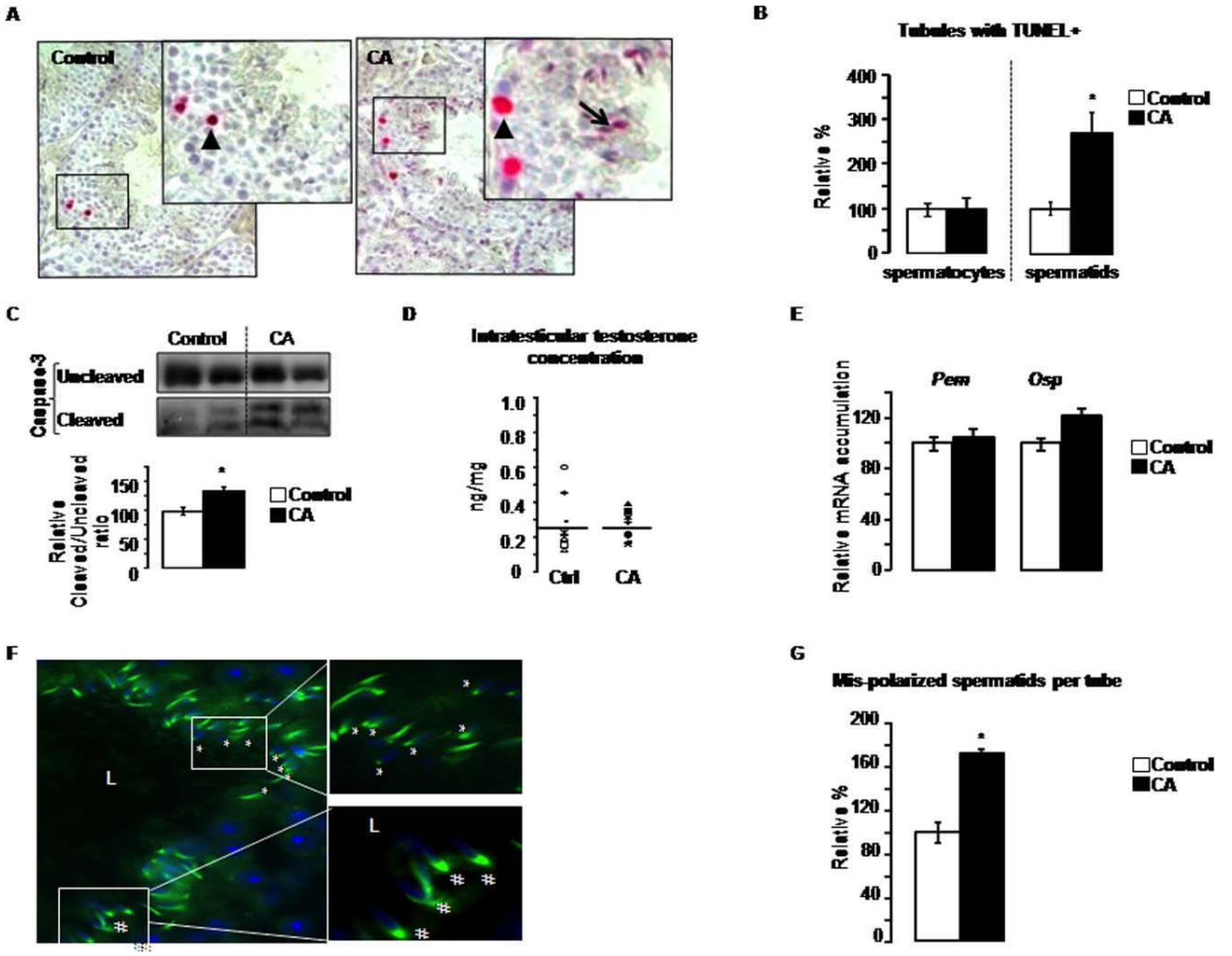

Fig. 2. CA diet induces germ cell apoptosis. (A) Apoptosis in mice exposed to control or CA diets (n = 10-20 per group) analyzed by TUNEL staining. Representative micrographs of the testis exposed to control or CA diets. Arrowheads indicate apoptotic spermatocytes; arrow indicates apoptotic spermatids. The original magnification was 200×. (B) Quantification of TUNEL analyses. The number of TUNEL-positive spermatocytes is indicated as the number of positive cells per 100 seminiferous tubules; the number of tubules with TUNEL-positive spermatids is indicated as the number of positive tubes per 100 seminiferous tubules (n = 10-20). Control-diet-treated mice were arbitrarily fixed at 100%. (C) Immunoblotting of cleaved caspase-3 performed on testicular protein extracts of mice fed a control or CA diet (n = 6-10 per group). Quantification of cleaved caspase-3 protein accumulation, compared to uncleaved caspase-3. Control-diet-treated mice were arbitrarily fixed at 100%. (D) IT testosterone concentration (ng/mg) in C57Bl/6J mice fed a control or CA diet for 4 months (n = 6-20 per group). (E) Testicular mRNA expression of *Pem* and *Osp* normalized to *β-actin* levels in whole testis of C57Bl/6J mice fed control or CA diet for 0.5 or 4 months (n = 10-15 per group). (F) Spermatid orientation, as measured by the stained cells for E-cadherin. Representative micrographs of mice fed 4 months with CA diet (n = 10-15 per group). #Indicates well-oriented spermatids; *indicates misoriented spermatids. The original magnification was 100×. (G) Quantification of the number of tubules with misoriented spermatids per 100 seminiferous tubules after 4 months of control or CA diet (n = 10-15 per group). Control-diet-treated mice were arbitrarily fixed at 100%. L, lumen. In all of the panels, data are expressed as the means ± standard error of the mean. Statistical analysis: $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

misoriented spermatids in $Tgr5^{-/-}$ males after BA exposure (Fig. 4D). In addition, $Tgr5^{-/-}$ mice exposed to a CA diet did not display decreased N-cadherin and Cx43 protein levels (Fig. 4E,F). Regarding *Cx43*, the protective effect of the lack of *Tgr5* was also observed at the mRNA level (Supporting Fig. 1F).

The Testicular Phenotype Is Not Initiated by Altered Liver Injury or Testosterone/Insulin-Like Growth Factor 1 Levels. The effects of prolonged BA exposure on male reproductive functions are dependent on the BA receptor TGR5, because $Tgr5^{-/-}$ mice are protected against the observed deleterious testicular effects. One could have hypothesized that differences in the extent of liver injury, induced by chronic CA feeding, contribute to the improved testicular physiology in $Tgr5^{-/-}$ males. However, consistent with a previous study,[25] body and liver weight were not different between $Tgr5^{+/+}$ and $Tgr5^{-/-}$ males in response to BA exposure (Supporting Fig. 2A). Furthermore, plasma alanine aminotransferase

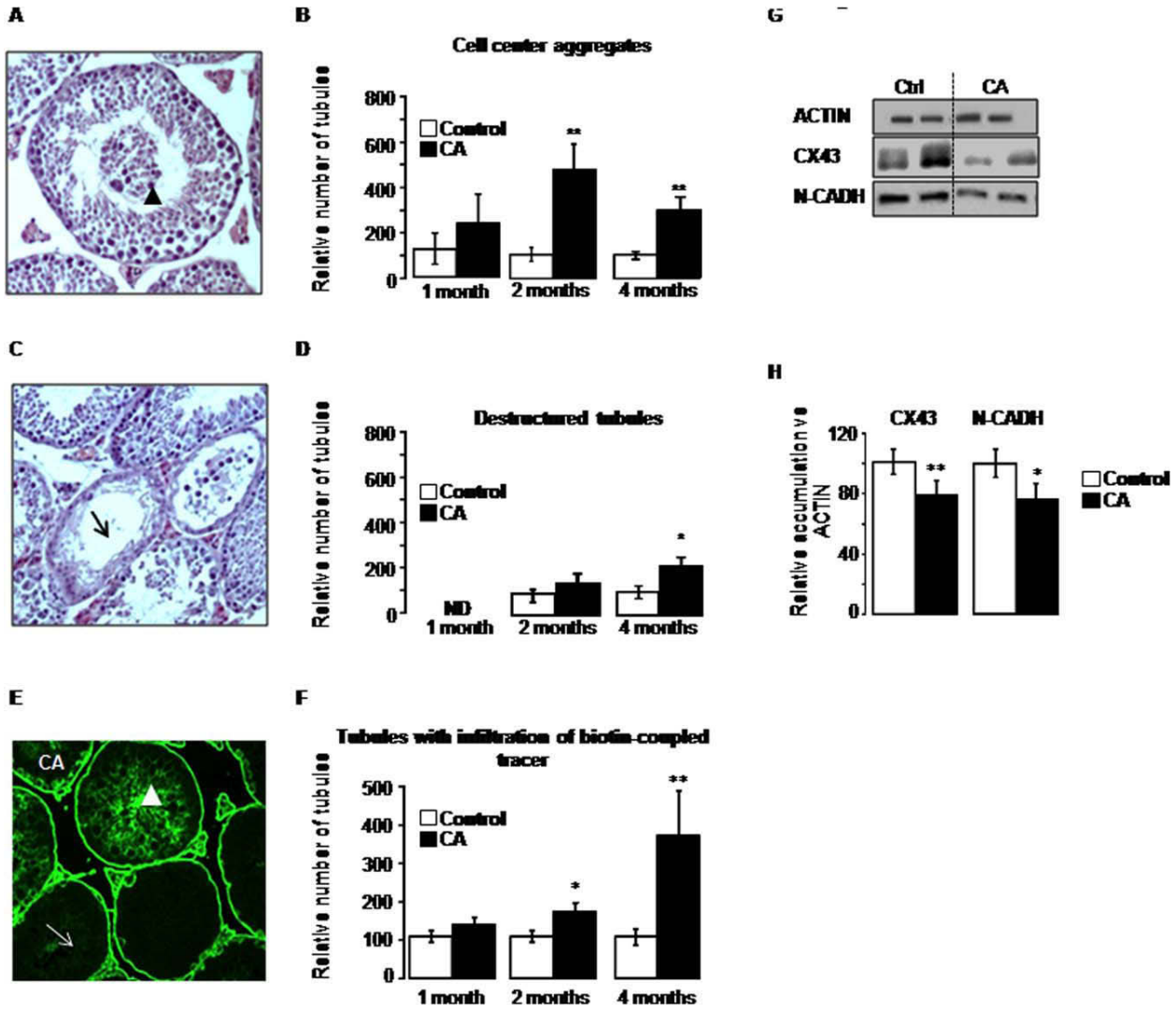

Fig. 3. CA diet impairs seminiferous epithelium integrity through the down-regulation of *connexin-43 and N-cadherin*. (A) Representative micrographs of H&E-stained testes of mice fed a CA diet for 4 months (n = 10-20 per group). Arrowhead indicates tubules with sloughing germ cells. The original magnification was 100×. (B) Quantification of the number of tubules with sloughing germ cells tubules per 100 seminiferous tubules after 1, 2, and 4 months of control or CA diets (n = 10-20 per group). (C) Representative micrographs of H&E-stained testes of mice fed a CA diet for 4 months (n = 10-20 per group). Arrows indicate tubes with complete loss of germ cells. The original magnification was 100×. (D) Quantification of the number of completely destructured tubules per 100 seminiferous tubules after 1, 2, and 4 months of control or CA diets (n = 10-20 per group). (E) BTB integrity, as measured by the stained testes for EZ-link biotinylated. Representative micrographs of mice fed 4 months with a control diet or CA diet (n = 10-15 per group). Arrow indicates a tubule with a slight infiltration of EZ-link biotinylated; arrowhead indicates a tubule with a high intensity of infiltration. The original magnification was 100×. (F) Quantification of the number of tubules with infiltration per 100 seminiferous tubules after 1, 2, and 4 months of a control or CA diet (n = 10-15 per group). (G) Immunoblottings of connexin-43 and N-cadherin protein accumulations, compared to actin, performed on testicular protein extracts of mice fed a control or CA diet for 2 months (n = 10-20 per group). (H) Quantification of connexin-43 and N-cadherin protein accumulations, compared to actin. In all panels, data are expressed as the means ± standard error of the mean. Statistical analysis: $*P < 0.05$ and $**P < 0.01$ versus control diet group. In all panels, control-diet–treated mice were arbitrarily fixed at 100%.

(ALT) levels and cytokeratin-8 were similarly increased in both genotypes after CA diet exposure (data not shown), suggesting a similar extent of liver injury in both genotypes.

Reduced plasma testosterone levels have been associated with liver diseases.[26,27] In our experimental model, lower testosterone levels were initially observed 1 month after initiation of the CA diet (Fig. 5A). This was also reflected by the lower weight of the seminal vesicles (Fig. 5B), which is an androgen-dependent organ. The lower plasma testosterone levels appear to be correlated with altered hepatic catabolism, as suggested by the higher mRNA accumulation of genes such as UDP glucuronosyltransferase 2 family,

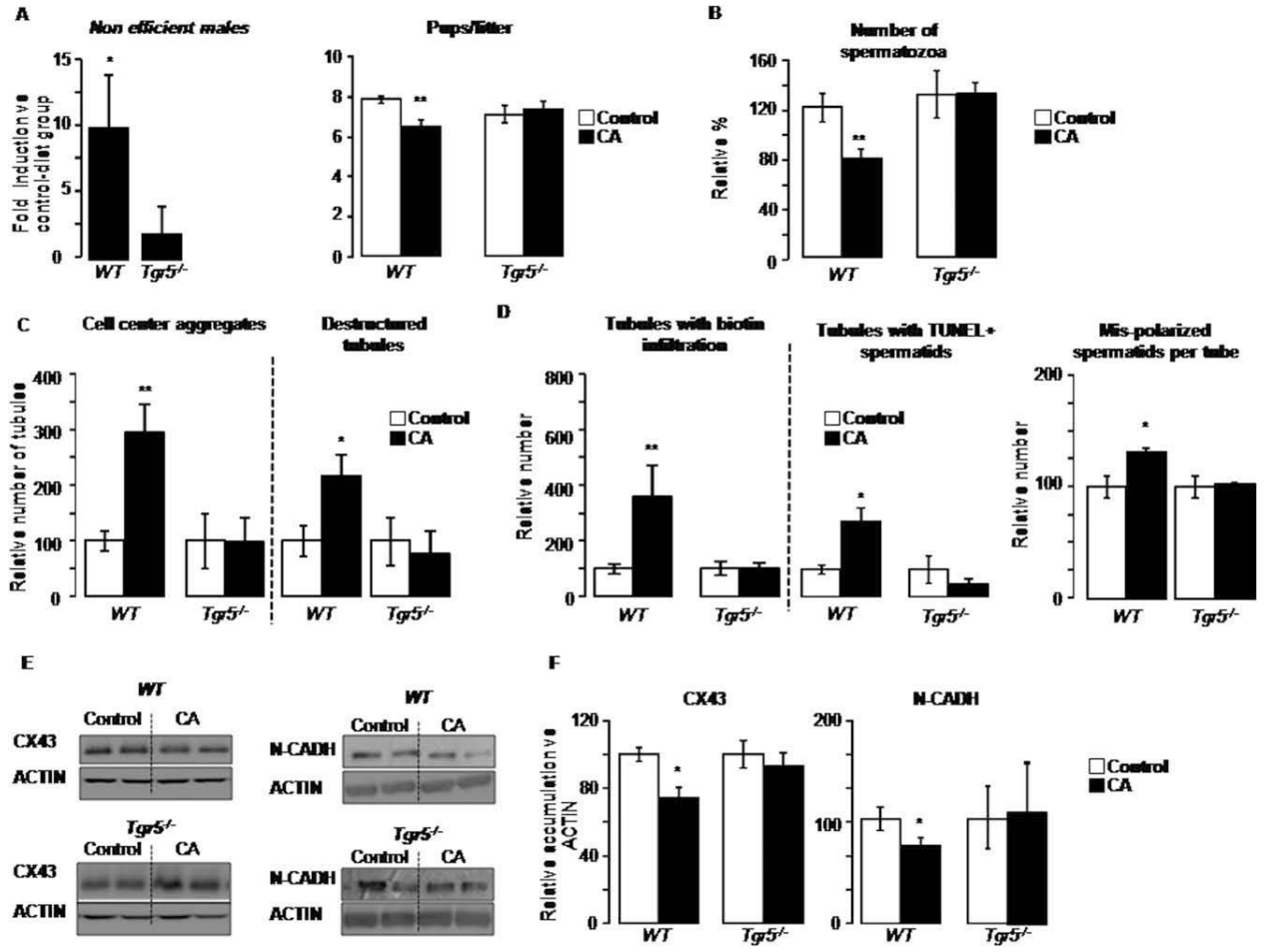

Fig. 4. *Tgr5* deficiency protects male mice from CA-diet–induced testicular abnormalities. (A) After 4 months of diet, each male of either WT or *Tgr5*$^{-/-}$ genotype was bred with 2 C57Bl/6J females to analyze their reproductive efficiency (fold increase of number of infertile males vs. control diet group) and the number of pups per litter (n = 6-10 per group); data are expressed as the means ± standard error of the mean. *Denotes significant difference from the control diet group for same genotype (*$P < 0.05$ and **$P < 0.01$). (B) Spermatozoa count in the tail of epididymis of WT or *Tgr5*$^{-/-}$ males exposed to 4 months of a control or CA diet (n = 10-20 per group). Control diet groups were arbitrarily fixed at 100% (**$P < 0.01$). (C) Quantification of the number of tubules with sloughing germ cells or completely destructured tubules per 100 seminiferous tubules in WT and *Tgr5*$^{-/-}$ mice fed a control or CA diet for 4 months (n = 6-10 per group). Control-diet–treated mice were arbitrarily fixed at 100%. *$P < 0.05$; **$P < 0.01$. (D) Quantification of the number of tubules with biotin infiltration; quantification of the number of tubules with TUNEL-positive spermatids; and quantification of the number of tubules with misoriented spermatids. For all parameters, 100 seminiferous tubules per mice were analyzed after 4 months in WT and *Tgr5*$^{-/-}$ mice fed a control or CA diet (n = 6-15 per group). Data are expressed as the means ± standard error of the mean. *Denotes significant difference from the control diet group for the same genotype (*$P < 0.05$ and **$P < 0.01$). (E) Immunoblotting of Cx43 and N-cadherin performed on testicular protein extracts of WT and *Tgr5*$^{-/-}$ mice fed a control or CA diet for 2 months (n = 10-15 per group). (F) Quantification of Cx43 and N-cadherin protein accumulations, compared to actin. Data are expressed as the means ± standard error of the mean. *Denotes significant difference from the control diet group for the same genotype ($P < 0.05$).

polypeptide B34 (*Ugt2b34)*, cytochrome P450, family 3, subfamily a, polypeptide 11 (*Cyp3a11*; Fig. 5C), and sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1 (*Sult2a1*; data not shown). However, the reduction of plasma testosterone after CA exposure may not be critically involved in the altered fertility and sperm production, as it was also observed in *Tgr5*$^{-/-}$ males (Fig. 5A). This result was further supported by the effect of the CA diet on seminal vesicles in *Tgr5*$^{-/-}$ males (Fig. 5B). In addition, no effect of the CA diet or *Tgr5* genotype was observed on other hormones, such as DHT, 3α-androstandiol, androsterone, or estradiol (Supporting Fig. 2B).

In addition, decreased levels of hepatic insulin-like growth factor 1 (*Igf1)* mRNA, a factor described to alter the BTB and a known inducer of *Cx43* expression, were also observed in WT males exposed to a CA diet. This decrease was observed from 15 days after beginning of BA exposure (Fig. 5D), a time when Cx43 was already decreased at the mRNA level

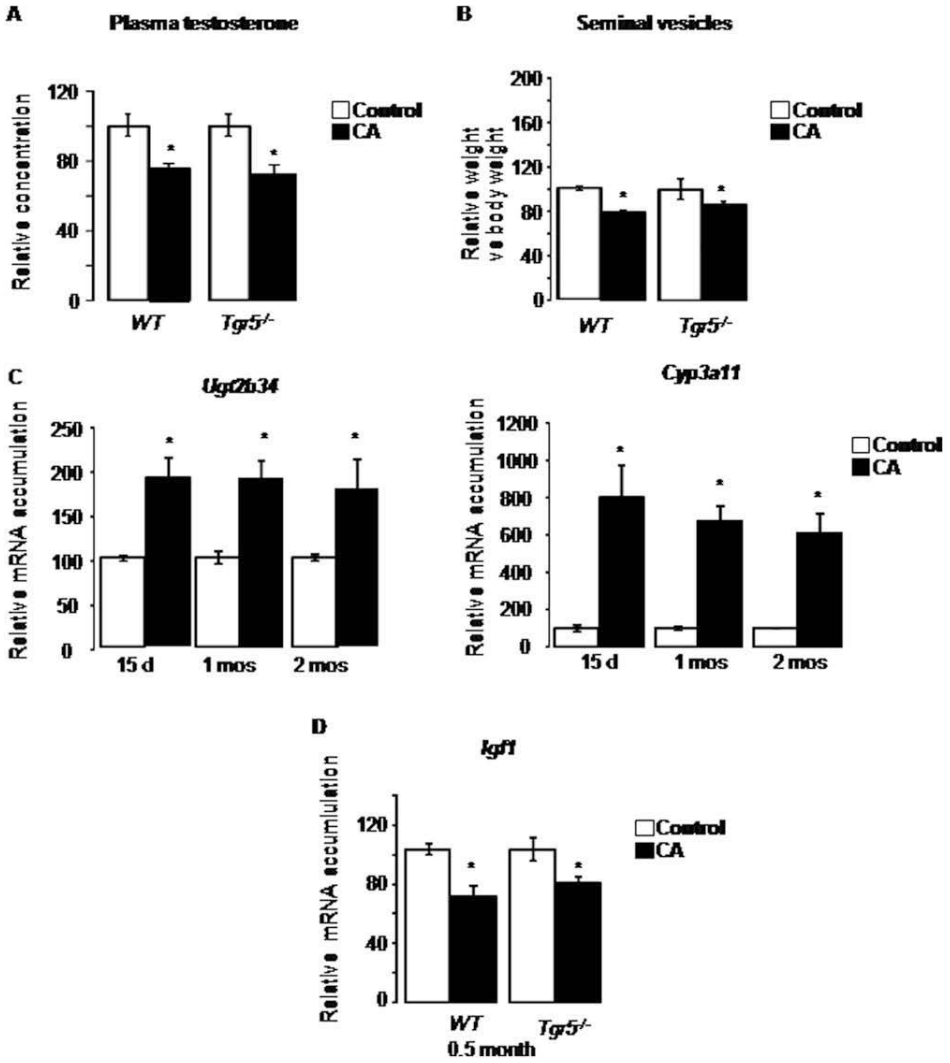

Fig. 5. Systemic effects are not involved in fertility disorders mediated by Tgr5. (A) Relative plasma testosterone levels in WT and *Tgr5*$^{-/-}$ mice fed a control or CA diet for 1 month (n = 6-10 per group). (B) Seminal vesicle weights normalized to body weight in WT and *Tgr5*$^{-/-}$ mice fed a control or CA diet for 1 month (n = 6-20 per group). (C) Hepatic mRNA expression of *Ugt2b34* and *Cyp3a11* normalized to *β-actin* mRNA levels in whole testis of WT and *Tgr5*$^{-/-}$ mice fed a control or CA diet for 0.5 month (n = 6 to 15 per group; *$P < 0.05$). (D) Hepatic mRNA expression of *Igf1* normalized to *β-actin* mRNA levels in WT and *Tgr5*$^{-/-}$ mice fed a control or CA diet for 15 days (n = 6 per group). In all panels, control diet groups were arbitrarily fixed at 100%. In all panels, data are expressed as means ± standard error of the mean. Statistical analysis: *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.

(Suppl. 1E). Interestingly, *Tgr5*$^{-/-}$ males displayed a similar alteration of *Igf1* mRNA accumulation after CA diet exposure (Fig. 5D). These results suggest that *Igf1* is not critically involved in the initiation of the reproductive disorders.

The Effect on Cell-Cell Interactions Is Mediated, in Part, Through Activation of TGR5 in Germ Cells by Testicular Bas. We then analyzed whether BAs can directly act through TGR5 in the testis. Using a fluorescein-coupled BA (Fig. 6A),[29] we observed that BAs were able to reach the testis by blood circulation and were mainly localized in the interstitial compartment in mice with an intact BTB. In contrast to males of the control group, males fed for 4 months with a CA diet displayed fluorescence within the seminiferous epithelium (Fig. 6B). Using MS, we demonstrated that BA levels were increased in WT males exposed to a CA diet (Fig. 6C). A similar increase was observed in *Tgr5*$^{-/-}$ males after BA exposure (Supporting Fig. 3A). Altered fertility was correlated with IT levels of DCA (Fig. 6D).

Because of the lack of reliable Abs to detect mouse TGR5 in testes by IHC, we analyzed the expression pattern of testicular *Tgr5* using the classical ontogeny approach. Unlike the somatic markers, *Fshr* (SCs) and *Lhcgr* (Leydig cells), whose expression peaked at 5 and 25 dpn, respectively, *Tgr5*, as well as the germ cell marker,

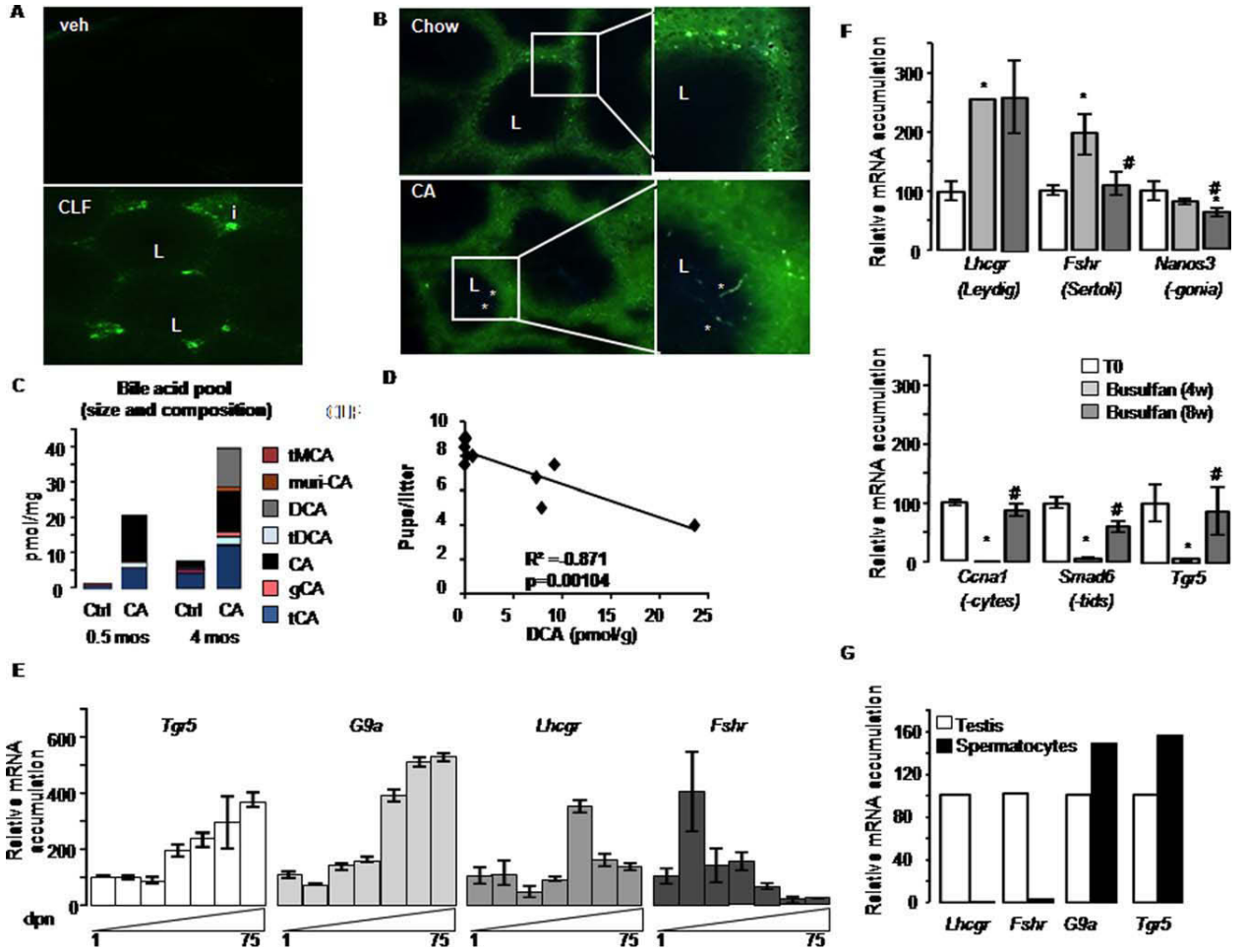

Fig. 6. Intratesticular BAs and expression of *Tgr5* in germ cells support the hypothesis of an active signaling pathway in the testis. (A) Analyses of BA localizations in the testis, as monitored by the IP injection of cholyl-lysyl-fluorescein (CLF; 0.6 mg/mice) or vehicle (phosphate-buffered saline [PBS] 1×) for 30 minutes. Representative micrographs of the stained testes of PBS- or CLF-injected mice. The original magnification was 200×. (B) Analyses of BA localizations in the testis, as monitored by IT injection of CLF (0.6 mg/mice) for 30 minutes in mice fed a control or CA diet for 4 months. Representative micrographs of the stained testes of PBS- or CLF-injected mice. The original magnification was 200×. (C) IT BA pool size and composition in mice fed a control or CA diet for 0.5 or 4 months (n = 5 per group). (D) Correlation of the number of pups per litter with the concentration of IT DCA concentration. (E) Testicular mRNA accumulation of *Tgr5*, *G9a*, *Lhcgr*, and *Fshr* normalized to *β-actin* mRNA levels in whole testes of C57BL/6J from 1 to 75 days old. Data are expressed as the means ± standard error of the mean. (F) Testicular mRNA accumulation of *Lhcgr*, *Fshr*, *Nanos*, *Ccna1*, *Smad6*, and *Tgr5* normalized to *β-actin* mRNA levels in whole testes of C57BL/6J mice treated with busulfan (20 mg/kg, one injection IP) at T0, 4, or 8 weeks (n = 8 per group). Data are expressed as means ± standard error of the mean. *Denotes significant difference from the T0 time point; #denotes significant difference from the 4-week time point ($P < 0.05$). (G) mRNA accumulation of *Lhcgr*, *Fshr*, *G9a*, and *Tgr5* normalized to *β-actin* levels in purified spermatocytes of adult C57BL/6J mice.

G9a, progressively accumulated over time (Fig. 6E). This may point to the possibility that *Tgr5* is expressed in germ cells. We also analyzed *Tgr5* in a model of germ cell loss (Fig. 6F and Supporting Fig. 3B).[24] Germ cell loss induced by busulfan exposure and subsequent recolonization of the epithelium were confirmed by analyzing the weight and histology of the testis (Supporting Fig. 2B) as well as by analyzing specific markers of Sertoli (*Fshr*), Leydig (*Lhcgr*), and germ cells at different steps of spermatogenesis (*Nanos*, *Ccna1*, and *Smad6*; Fig. 6F). The expression pattern indicated that *in vivo* *Tgr5* is mainly expressed in germ cells (Fig. 6F). In addition, as for the germ cell marker, *G9a*, *Tgr5* mRNA expression was detected in purified spermatocytes (Fig. 6G).

To determine whether BAs can act directly on germ cells through TGR5, we exposed the germ cell line, GC1spg, to a panel of TGR5 agonists, including LCA,[30] OA,[31] and DCA[30] (Fig. 7A). Interestingly, all agonists repressed Cx43 protein accumulation at concentrations that were in line with the reported EC values (Fig. 7A). Moreover, introduction of a specific siRNA directed against *Tgr5* (Supporting Fig. 4A) abolished the reduction of *Cx43* by DCA (Fig. 7B). Moreover, in untreated cells, *Tgr5* silencing induced

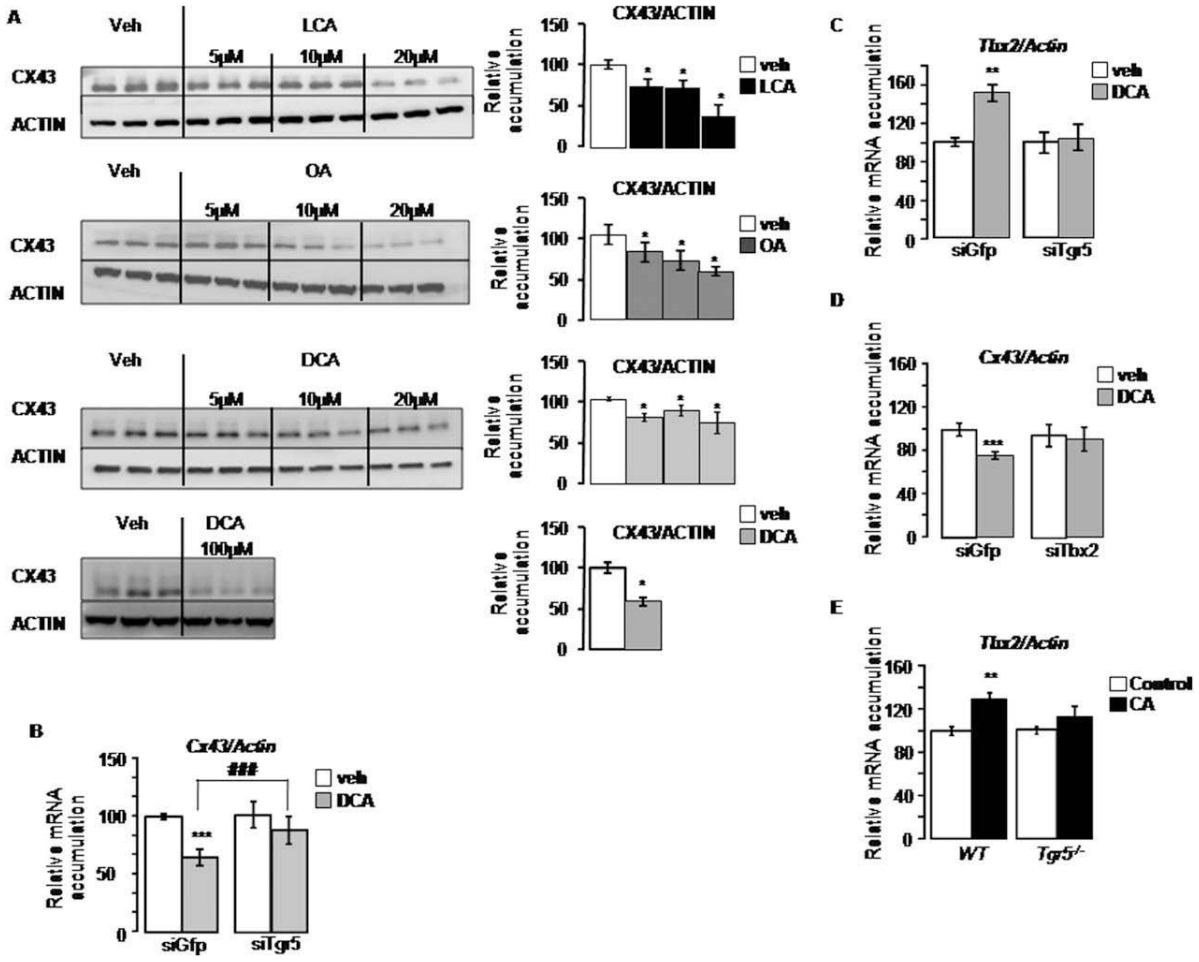

Fig. 7. TGR5 controls *Cx43* accumulation through a TBX2 pathway. (A) Cx43 protein accumulation levels normalized to actin in the GC1spg germ cell line exposed to vehicle (ethanol) or DCA, LCA, or OA over 24 hours (n = 18 per group). Vehicle-treated cells were arbitrarily fixed at 100%. (B) mRNA expression of *Cx43* normalized to *β-actin* levels in GC1spg germ cell lines transfected with siGfp or siTgr5 and exposed to vehicle or DCA during 24 hours (n = 15 per group). *Denotes difference from the siGfp vehicle group; #denotes difference from the siGfp DCA group. siGfp vehicle-treated cells were arbitrarily fixed at 100% for each siRNA condition. (C) mRNA expression of *Tbx2* normalized to *β-actin* levels in GC1spg germ cell lines transfected with siGfp or siTgr5 and exposed to vehicle or DCA during 24 hours (n = 15 per group). Vehicle-treated cells were arbitrarily fixed at 100% for each siRNA condition. (D) mRNA expression of *Cx43* normalized to *β-actin* levels in GC1spg germ cell line transfected with siGfp or siTbx2 and exposed to vehicle or DCA over 24 hours (n = 12 per group). Vehicle-treated cells were arbitrarily fixed at 100% for each siRNA condition. (E) Testicular mRNA expression of *Tbx2* normalized to *β-actin* mRNA levels in whole testes of WT and *Tgr5*$^{-/-}$ mice fed a control or CA diet for 15 days (n = 12 to 20 per group). Control diet groups were arbitrarily fixed at 100%. In all panels, data are expressed as means ± standard error of the mean. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.

an increase of Cx43 mRNA accumulation, compared to siGfp (Suppl. 4B).

The *Cx43* gene has been reported to be repressed by several transcription factors, including TBX2.[33] Consistent with the kinetics of *Cx43* regulation, *Tbx2* expression was induced 6 hours after DCA exposure (Supporting Fig. 4C) and remained increased up to 24 hours (Fig. 7C). Induction of *Tbx2* mRNA was absent in cells transfected with siTgr5, compared with cells transfected with control siGfp (Fig. 7C). Moreover, involvement of TBX2 was confirmed through use of a specific siRNA directed against *Tbx2* (Supporting Fig. 4D) that prevented the effects of DCA on *Cx43* expression (Fig. 7D). Consistent with these cell-based studies, *Tbx2* mRNA also accumulated *in vivo* in the testis after 2 weeks of the CA diet (Fig. 7E), whereas Cx43 mRNA accumulation decreased (Supporting Fig. 1E). Moreover, these effects were lost in *Tgr5*$^{-/-}$ mice (Fig. 7E), supporting the hypothesis of a DCA-TGR5-Tbx2-Cx43 signaling axis.

Discussion

Here, we demonstrated that chronic exposure to elevated BA levels leads to reduced male fertility along with significantly lower production of spermatozoa. We demonstrated that mice fed a diet supplemented

with CA exhibit germ cell sloughing and BTB rupture, as well as apoptosis of spermatids, and that these effects are reversed in mice deficient for *Tgr5*. These results strongly indicate that constitutively active BA-TGR5 signaling may alter testicular epithelium integrity. The morphological abnormalities, exemplified by cell detachment and destructured tubules, that are observed after long-term treatment with BAs are, in fact, highly reminiscent of the infertility observed in humans and mouse models after portacaval shunting[34] or exposure to endocrine disruptors,[35,36] suggesting that these alterations might be involved in the observed altered reproductive functions induced by the CA diet.

Although CA-fed animals displayed lower plasma testosterone levels, most likely the result of altered hepatic catabolism of testosterone, the lack of any effect of CA exposure on testicular testosterone synthesis was rather unexpected. We previously reported that the BA-responsive nuclear receptor, FXR-α, represses testicular steroidogenesis after short exposure to FXR-α synthetic agonists.[9] This apparent discrepancy might be related to the difference in the potency of CA diet and GW4064 in activating FXR-α or, alternatively, may depend on duration of treatment, which was significantly different between both studies.[9] Furthermore, the absence of any major difference in IT testosterone synthesis was consistent with our germ cell phenotype. In fact, it has been well established that a decrease in IT testosterone triggers apoptosis during the stage of spermatocyte development,[20] whereas, in our study, apoptosis was detected in spermatids.

Our study revealed that *Tgr5* is expressed within the germ cell lineage, where it represses *Cx43* expression through regulation of the transcriptional repressor, *Tbx2*. Numerous studies have highlighted the importance of Cx43 and N-cadherin in testicular physiology to maintain the number of germ cells.[37-40] Based on our findings, we propose that the concomitant decrease of Cx43 and N-cadherin may account for germ cell sloughing and BTB alteration after BA exposure. This function of TGR5 in germ cells is consistent with recent studies indicating that interactions between SCs and germ cells (spermatids) can perturb BTB integrity.[5,6] Moreover, animals treated with BAs presented incorrectly oriented spermatids, which could also be associated with spermatid apoptosis.[7]

Even though systemic factors, such as testosterone and IGF-1, can, in theory, induce male infertility, our data rather disfavor this possibility in our model. Instead, the results obtained from our studies in $Tgr5^{-/-}$ males support the idea that BAs could directly affect testicular physiology through TGR5 in germ cells. Indeed, we observed that BAs are present in the testis and that their concentrations are increased under CA-diet exposure. Such an increase was observed in both $Tgr5^{+/+}$ and $Tgr5^{-/-}$ males. This suggests that the lower response to the CA diet in the testes of $Tgr5^{-/-}$ mice in terms of N-cadherin and Cx43 protein accumulation was not the result of altered BA levels, but rather the absence of an active TGR5-signaling pathway. It is interesting to note that DCA is the BA displaying the highest fold increase after CA-diet exposure in the testes. Of all naturally occurring BAs, DCA is one of the most potent agonists for TGR5.[30,31] This finding is consistent with a role for TGR5 in the CA-induced deleterious effects on testis physiology. DCA is a secondary BA that originates from the intestinal transformation of CA by bacterial flora, suggesting that an intact enterohepatic cycle is required to induce the testicular-deleterious effects. Intriguingly, DCA plasma and IT concentrations were negatively correlated with the number of pups per litter from CA-exposed males, further corroborating the importance of DCA in this process. Additional studies will be necessary to confirm whether DCA may have similar effects on male fertility and function in humans.

Our work suggests a key role for TGR5 within the testis where TGR5 may potentially be activated by BAs in pathological conditions with increased BA levels, but the physiological ligands remain to be defined. However, it is interesting to note that TGR5 has been demonstrated to be activated by some steroids that may be relevant in testicular physiology, such as androsterone and androstandiol, which may be natural testicular ligands of TGR5.[31] More work will be required to assess the contribution of these steroids to TGR5 function during testicular development and physiology. Finally, it is worth mentioning that several correlations exist in humans between liver diseases and altered male fertility.[41,42] Given that many liver diseases are characterized by a severely impaired BA homeostasis, it is tempting to speculate that pathological BA levels in serum may initiate testicular alterations during the early stages of liver dysfunction and subsequently promote subfertility. In this context, plasma BA concentrations and pool composition may offer promising tools for early diagnosis of testicular subfertility in patients with liver diseases. Although more human studies are warranted to corroborate this correlation in humans, our mouse studies provide strong indications for a deleterious effect of chronic BA exposure on testicular pathophysiology and fertility disorders.

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Supporting Information

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Hepatotoxicity induced by neonatal exposure to diethylstilbestrol is maintained throughout adulthood via the nuclear receptor SHP.

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Expert Opinion, 2014

EXPERT OPINION

Hepatotoxicity induced by neonatal exposure to diethylstilbestrol is maintained throughout adulthood via the nuclear receptor SHP

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Background: Liver physiology is sensitive to estrogens, which suggests that the liver might be a target of estrogenic endocrine disrupters (EED). However, the long-term consequences of neonatal exposure to EED on liver physiology have rarely been studied. The nuclear receptor small heterodimer partner (SHP) mediates the deleterious effects of neonatal exposure to diethylstilbestrol (DES) on male fertility.
Objectives: As SHP is involved in liver homeostasis, we aimed to determine whether neonatal estrogenic exposure also affected adult liver physiology through SHP. Male mouse pups were exposed to DES in the first 5 days of life.
Results: DES exposure leads to alterations in the postnatal bile acid (BA) synthesis pathway. Neonatal DES-exposure affected adult liver BA metabolism and subsequently triglyceride (TG) homeostasis. The wild-type males neonatally exposed to DES exhibited increased liver weight and altered liver histology in the adult age. The use of deficient male mice revealed that SHP mediates the deleterious effects of DES treatment. These long-term effects of DES were associated with differently timed alterations in the expression of epigenetic factors.
Conclusions: However, the molecular mechanisms by which neonatal exposure persist to affect the adult liver physiology remain to be defined. In conclusion, we demonstrate that neonatal DES exposure alters adult hepatic physiology in an SHP-dependent manner.

Keywords: estrogenic endocrine disrupter, liver, small heterodimer partner

Expert Opin. Ther. Targets [Early Online]

1. Introduction

The small heterodimer partner (SHP, also known as NR0B2) is a member of the nuclear receptor (NR) superfamily and has been demonstrated to play important roles in liver physiology. SHP is primarily known for its functions in the regulation of bile acid (BA) homeostasis. This role was unequivocally established by the characterization of *Shp*-deficient mouse models [1,2]. Additionally, SHP also regulates hepatic gluconeogenesis [3,4] and lipid metabolism [5]. SHP is an atypical NR that lacks a DNA-binding domain [6] whose functions have been linked to its ability to repress the transcriptional activity of other NRs such as the liver homolog-1 (LRH-1; NR5A2) [7] and the liver X receptorα (LXRα; NR1H3) [8]. Additionally, several lines of evidence have highlighted the crosslinks between SHP and the estrogen signaling pathways. SHP interacts and inhibits the transcriptional activity of the estrogen receptors (i.e., Erα/β and Nr3a1/2) [9]. Additionally, *Shp* is a direct target

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gene of both ERs [10]. Moreover, a portion of the estrogenic effects of diethylstilbestrol (DES) in the testis might be mediated through SHP [11].

The liver has been defined as a target of estrogenic signaling pathways. Estrogens have bivalent effects on liver physiology [12]. Some positive effects have been reported; for example, total estrogen receptor alpha (ER-α) knockout mice exhibit hepatic insulin resistance [13,14]. The loss of ER-α in the liver has also been associated with hepatic steatosis and inflammation [15]. Estrogens can inhibit CCl_4-induced hepatic injury through the induction of hepatic miR-29 [16]. Moreover, estrogens have a protective effect against the development of liver cancer [17]. However, deleterious effects of estrogens have also been reported; for example, estrogen have been associated with cholestasis pathology [18,19]. Estrogen-induced cholestasis is a common form of cholestasis syndromes [20]. Estrogen-induced hepatotoxicity in rodent models is mediated through hepatic ER-α [12]. Moreover, 5 days of exposure to supra-physiological doses of 17α-ethynylestradiol (EE2) altered synthesis pathways and uptake and efflux of BAs and cholesterol in mice [19,21].

In utero and/or early postnatal development has been demonstrated to be highly sensitive to exposure to estrogen-like EDs such as estradiol benzoate and DES [22]. To define the involvement of SHP in the effects of estrogenic EDs on liver physiology, wild-type ($Shp^{+/+}$) and *Shp* null ($Shp^{-/-}$) males were exposed to DES during early post-natal life. Here, we show that at early postnatal ages, DES exposure affected the BA synthesis pathway. Additionally, in adulthood, the DES-treated males exhibited increases in liver weight that were correlated with higher proliferation rates. Neonatal exposure to DES also affected adult BA metabolism and subsequently affected triglyceride (TG) homeostasis. These effects are mediated through the regulation of genes involved in BA synthesis, efflux, and/or catabolism. The persistence of neonatal exposure in adult physiology was associated with the altered expression of several epigenetic factors. These adult effects were mediated by SHP, as evidenced by the finding that the effects of DES treatment were less severe in the $Shp^{-/-}$ male mice.

2. Experimental procedures

2.1 Animals

The mice used have been previously described [7], were maintained on a mixed 129sv/C57BL/6J background (50 – 50%), and were housed in temperature-controlled rooms on a 12 h light/dark cycle. Based on the previous studies [19,11], the mice were treated (subcutaneous injection) on the first 5 days of life (5 µl per day) with vehicle (corn oil, MP Biomedicals, France) or DES (0.75 or 5 µg/pup/day; Sigma Aldrich, L'Isle d'Abeau, France). The mice were sacrificed either at postnatal 10 day or at 10-weeks old.

This study was conducted in accordance with the current regulations and standards approved by the Animal Care Committee (CEMEA Auvergne; based on the protocol CE 59-12). The protocol was performed as documented previously (28).

2.2 Histology

The livers were collected, formalin-fixed, and embedded in paraffin, and 5-µm-thick sections were prepared and stained with hematoxylin/eosin (n = 6 – 10 animals per group).

2.3 Immunohistochemistry

Paraffin sections of PFA-4% fixed liver were sectioned at 5 µm. The sections were mounted on positively charged glass slides (Superfrost plus), deparaffinized, rehydrated, treated for 20 min at 93 – 98°C in citric buffer (0.01 M, pH 6), rinsed in osmosed water (2 × 5 min), and washed (2 × 5 min) in Tris-buffered saline. Immunohistochemistry was conducted according to the manufacturer's recommendations as described earlier [23]. The slides were then counterstained with Hoestch medium (1 mg/ml). The Ki-67 antibody (M3064, Spring Bioscience, Pleasanton, CA) was used.

2.4 BA measurements

Liver BAs were analyzed in acetonitrile-deproteinized samples [24]. The qualitative measurements of the BAs in the liver extracts were performed using CrystalChem (Downers Grove, IL, USA) ELISA kits (Cat# 80470) following the manufacturer's recommendations.

2.5 Triglyceride measurements

The liver lipids were extracted as previously described [25]. The TGs were measured using a commercial kit (Diagnostic System, Holzheim Germany).

2.6 Real-time RT-PCR

RNA from the liver samples was isolated using Nucleospin RNA L (Macherey-nagel, Hoerdt, France). cDNA was synthesized from the total RNA with the MMLV reverse transcriptase and random hexamer primers (Promega, Charbonnière Les Bains, France). The real-time PCR measurements of the individual cDNAs were performed using SYBR green dye (Master mix Plus for SYBR Assay, Eurogentec, Angers, France) to measure the duplex DNA formation with the EppendorfRealplex system. The sequences of primers are reported in the supplemental data as Supplemental Table 1. Standard curves were generated with pools of liver cDNAs from the animals with different genotypes and/or treatments. The results were analyzed using the ΔΔct method.

2.7 Western blot

Proteins were extracted from the tissues using lysis buffer containing the following: 20 mM HEPES, 400 mM NaCl, 1 mM MgCl2, 200 µM EDTA in 25% de glycerol, and 1X protease inhibitors (Roche Diagnostics, Meylan, France). The antibodies used in this study were as follows: ACTIN (Sigma Aldrich A2066) and CYTOKERATINE-8 (CK8)

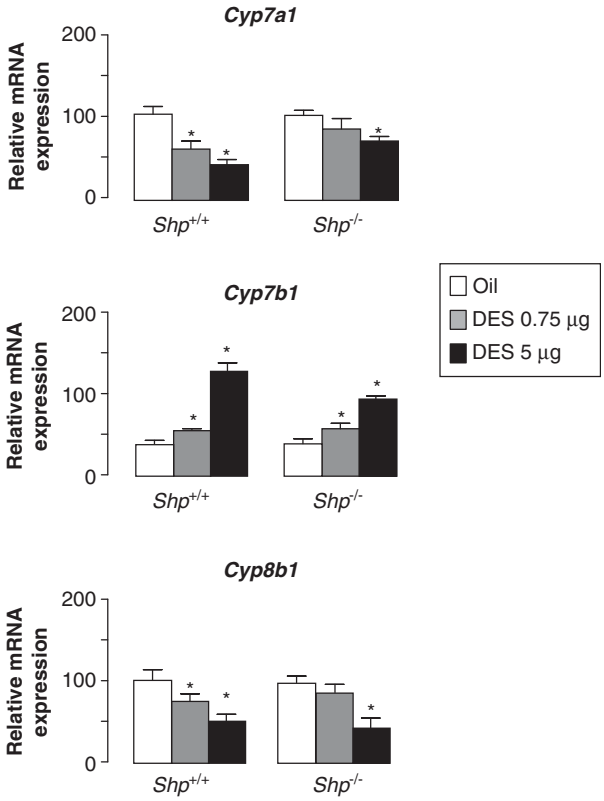

Figure 1. Neonatal exposure to DES alters expression of genes involved in bile acid synthesis. mRNA expression of *Cyp7a1*, *Cyp8b1*, and *Cyp7b1* normalized to *β-actin* levels in liver of 10 day-postnatal $Shp^{+/+}$ and $Shp^{-/-}$ mice exposed neonatally to vehicle, 0.75 µg or 5 µg of DES. mRNA expression of *Cyp7a1*, *Cyp8b1*, and *Cyp7b1* normalized to *β-actin* levels in liver of 10 day-postnatal $Shp^{+/+}$ and $Shp^{-/-}$ mice exposed neonatally to vehicle, 0.75 µg or 5 µg of DES. In all of the panels data are expressed as the means ± SEM. Statistical analysis: *p < 0.05 versus control diet group; n = 6 – 10 per group.
DES: Diethylstilbestrol.

(Santa-Cruz, sc-52324). These antibodies were used in TBS, 0.1% tween and 10% milk.

2.8 Statistics

For statistical analysis, 2-way ANOVAs were performed. When significant effects of treatment or genotype or their interactions were obtained, multiple comparisons were made with Tukey's test. All numerical data are presented as the means ± the SEMs. A p value < 0.05 was considered significant.

3. Results

3.1 Neonatal exposure to DES altered the BA synthesis pathway

The hepatotoxicity induced by estrogenic compounds in the adult has been associated with alterations of the expressions of genes involved in BA synthesis [19]. Consistent with these results, neonatal exposure to DES at both of the doses used induced decreases in mRNA accumulation of *Cyp7a1* and *Cyp8b1*, whereas the expression of *Cyp7b1* was increased (**Figure 1A**). Interestingly, these effects of neonatal DES exposure were less pronounced in the $Shp^{-/-}$males, as *Cyp7a1* and *Cyp8b1* were affected only at the dose of 5 µg of DES in these animals (**Figure 1A and B**).

3.2 Neonatal exposure to DES alters adult BA xenobiotic metabolism

Neonatal endocrine disrupter (ED) exposure has been demonstrated to induce abnormalities in adult that result in different pathologies such as infertility [26,27]. We wondered similar effects could be observed regarding liver physiopathology. Thus, we measured liver BA levels in response to DES exposure. The BA concentrations were significantly increased by both doses of DES in the $Shp^{+/+}$ mice but not in $Shp^{-/-}$ mice (**Figure 2A**). BA homeostasis could potentially be altered at several levels, including synthesis, uptake and/or efflux. Because the effects of DES on BA levels were observed with the lower dose used (0.75 µg), we decided to focus on this dose to decipher the involved mechanisms. Neonatal exposure to 0.75 µg of DES was associated with decreased expression of genes involved in BA synthesis and those encoding cytochromes *Cyp8b1*, *Cyp7b1*, and *Cyp27a1* (**Figure 2B**), whereas

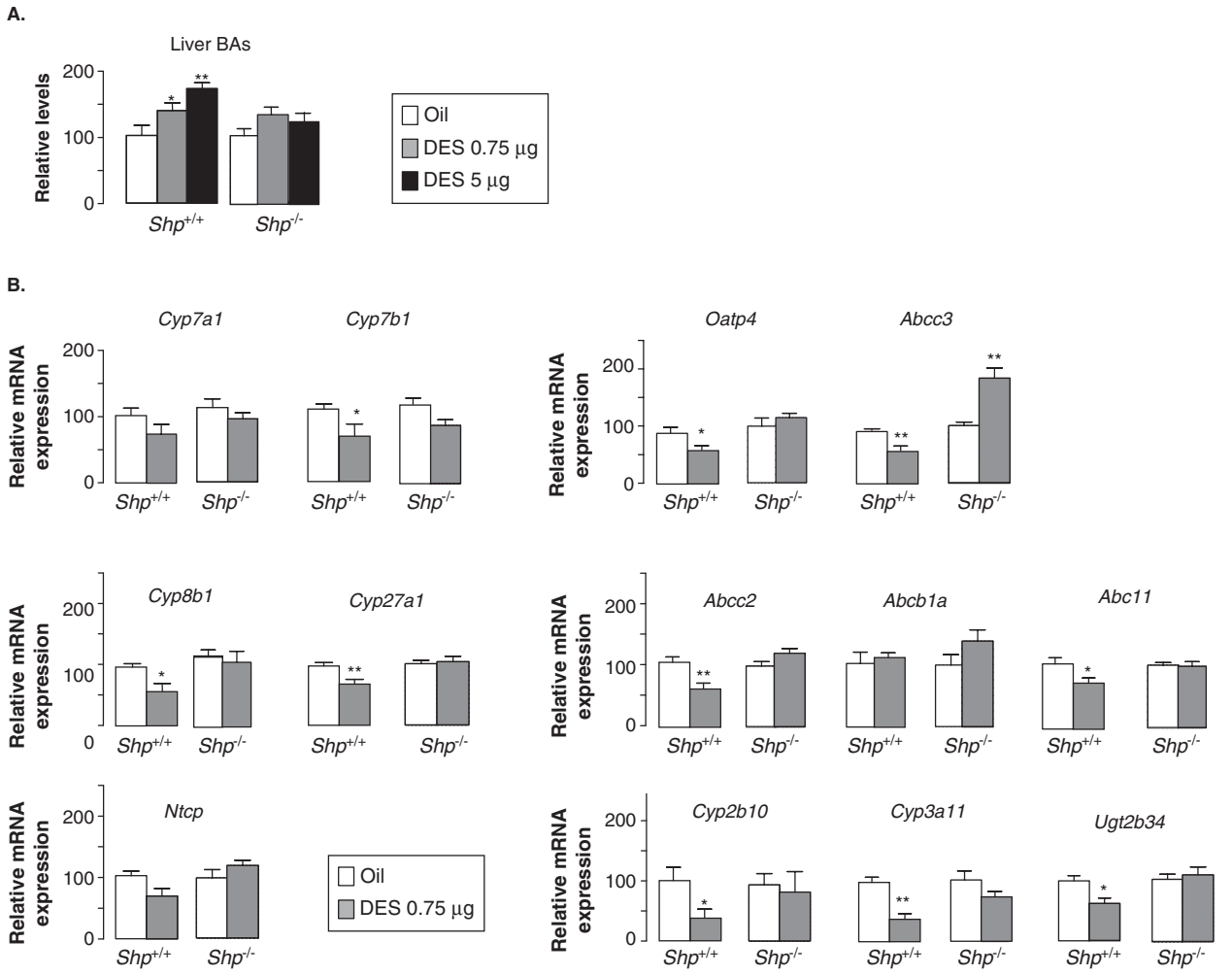

Figure 2. Neonatal exposure to DES alters adult bile acid metabolism. A. Liver relative concentrations of bile acids of adult *Shp*$^{+/+}$ and *Shp*$^{-/-}$ mice exposed neonatally to vehicle, 0.75 µg or 5 µg of DES. **B.** mRNA expression of *Cyp7a1, Cyp7b1, Cyp8b1, Cyp27a1, Ntcp, Oatp4, Abcc3, Abcc2, Abc1a, Bsep, Cyp2b10, Cyp3a11,* and *Ugt2b34* normalized to *β-actin* levels in liver of adult *Shp*$^{+/+}$ and *Shp*$^{-/-}$ mice exposed neonatally to vehicle or 0.75 µg of DES. In all of the panels data are expressed as the means ± SEM. Statistical analysis:*p < 0.05 versus control diet group; n = 6 – 10 per group.

no effect on *Cyp7a1* mRNA accumulation was observed (**Figure 2B**). No alterations were observed in terms of mRNA accumulation of the *Na+-taurocholate-cotransporting polypeptide* (*Ntcp*), which is a protein that is, involved in BA uptake (**Figure 2B**). Additionally, DES affected the efflux of BA either in the bile or in the circulation as evidenced by the decreased expressions of *Organic anion transporting polypeptide 4* (*Oatp4*), *ATP-binding cassette sub-family c member 3* (*Abcc3*), *Abcc2, Abcc1a, and Abcb11* (**Figure 2B**). Finally, BA and xenobiotic homeostases might also have compromised as shown by the accumulation of the mRNAs of genes involved in their catabolism. *UDP glucuronosyltransferase 2 family, polypeptide B34* (*Ugt2B34*), *Cyp3a4*, and *Cyp2b10* were decreased specifically in the wild-type males treated with DES (**Figure 2B**).

The main role of SHP in these alterations of metabolism following DES exposure is supported by the lack of effects on the liver BA levels and on most of the analyzed genes in the *Shp*$^{-/-}$ males (**Figure 2A and B**).

3.3 Neonatal exposure to DES alters TG metabolism. We then sought to define the effects of the increased hepatic BA levels

In the liver, TG synthesis has been defined to be a target of BA through the FXR-SHP-LXR-*Srepb1c* pathway [5]; thus, we analyzed TG homeostasis in livers exposed to DES. At the molecular level, the mRNA accumulations of *Diglyceride-acyltransferases 1 and 2* (*Dgat1/2*), *Fatty-acid synthase* (*Fasn*), *and Acetyl-CoA carboxylase* (*Acc*) were unchanged

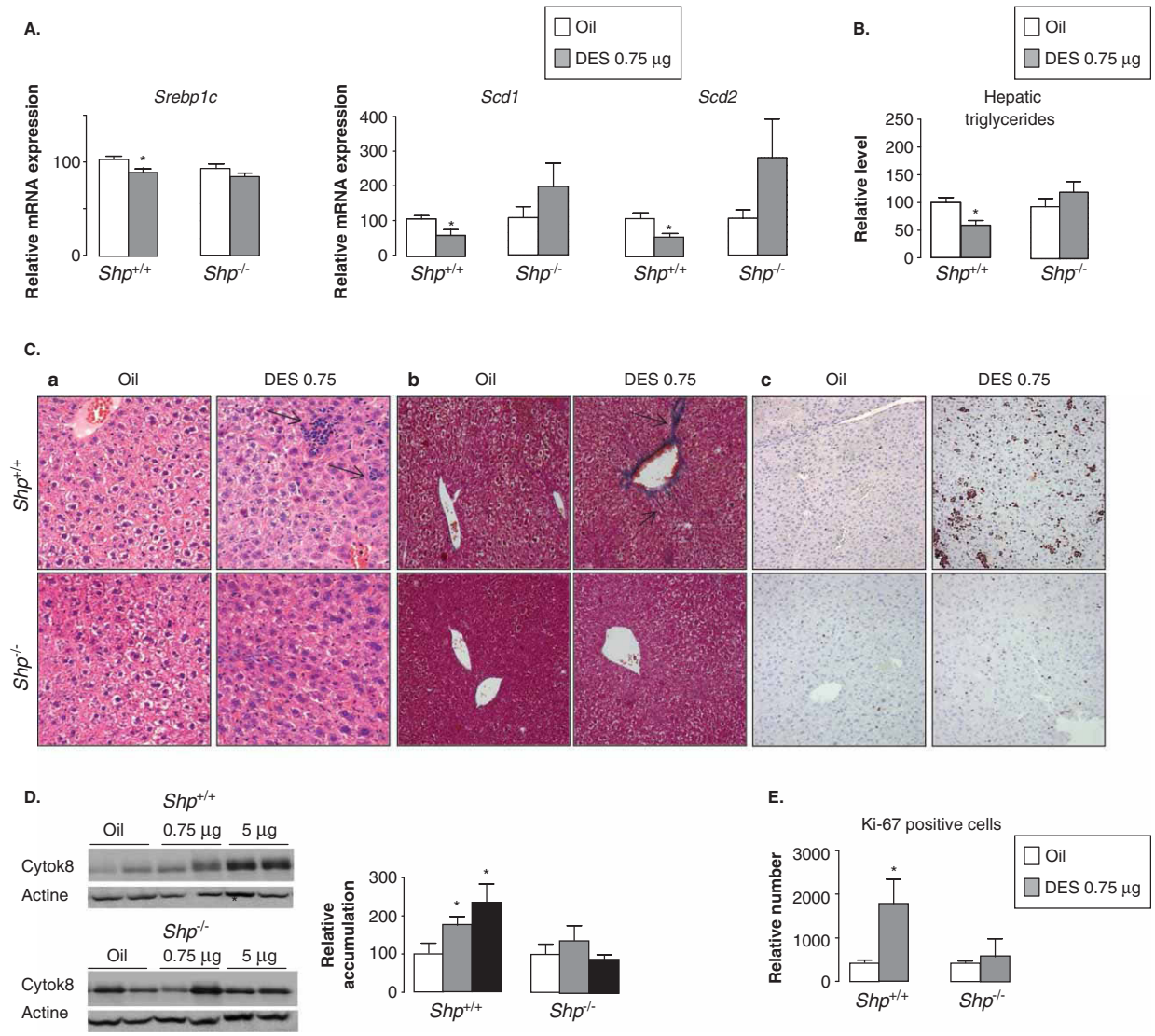

Figure 3. Neonatal exposure to DES alters adult TG metabolism. A. mRNA expression of *Srepb1c, Scd1,* and *Scd2* normalized to *β-actin* levels in liver of adult $Shp^{+/+}$ and $Shp^{-/-}$ mice exposed neonatally to vehicle or 0.75 µg of DES. **B.** Liver relative concentrations of TGs of adult $Shp^{+/+}$ and $Shp^{-/-}$ mice exposed neonatally to vehicle or 0.75 µg of DES. **C.** a: representative micrographs of hematoxylin/eosin-stained liver of adult $Shp^{+/+}$ and $Shp^{-/-}$ mice exposed neonatally to veh or 0.75 µg of DES. Arrows indicate lymphocytes infiltration. b: representative micrographs of Trichrome de asson-stained liver of adult $Shp^{+/+}$ and $Shp^{-/-}$ mice exposed neonatally to vehicle or 0.75 µg of DES. Arrows indicate fibrotic area. **c**: representative micrographs of Ki67-stained liver of adult $Shp^{+/+}$ and $Shp^{-/-}$ mice exposed neonatally to vehicle or 0.75 µg of DES. **D.** Immunoblot of CK8 protein accumulations compared to ACTIN performed on liver of adult $Shp^{+/+}$ and $Shp^{-/-}$ mice exposed neonatally to vehicle or 0.75 µg of DES. Quantification of CK8 protein accumulations compared to ACTIN. **E.** Quantification of the number of Ki-67 stained cells per slide in adult $Shp^{+/+}$ and $Shp^{-/-}$ mice exposed neonatally to vehicle or 0.75 µg of DES. In all of the panels data are expressed as the means ± SEM. Statistical analysis: *$p < 0.05$ versus control diet group; n = 6 – 10 per group.
CK8: Cytokeratine 8; DES: Diethylstilbestrol.

compared to the control group (data not shown). In contrast, the mRNA accumulations of *Sterol Regulatory Element-Binding Protein1c* (*Srebp1c*), *Stearoyl-coA-desaturase* 1 and 2 (*Scd1* and *Scd2*) were found to be decreased in the DES-treated $Shp^{+/+}$ animals (**Figure 3A**). The expressions of the studied genes were not altered following DES exposure in the $Shp^{-/-}$ males (**Figure 3A**). Consistent with the gene expression pattern, the $Shp^{+/+}$ mice exhibited reduced hepatic TG

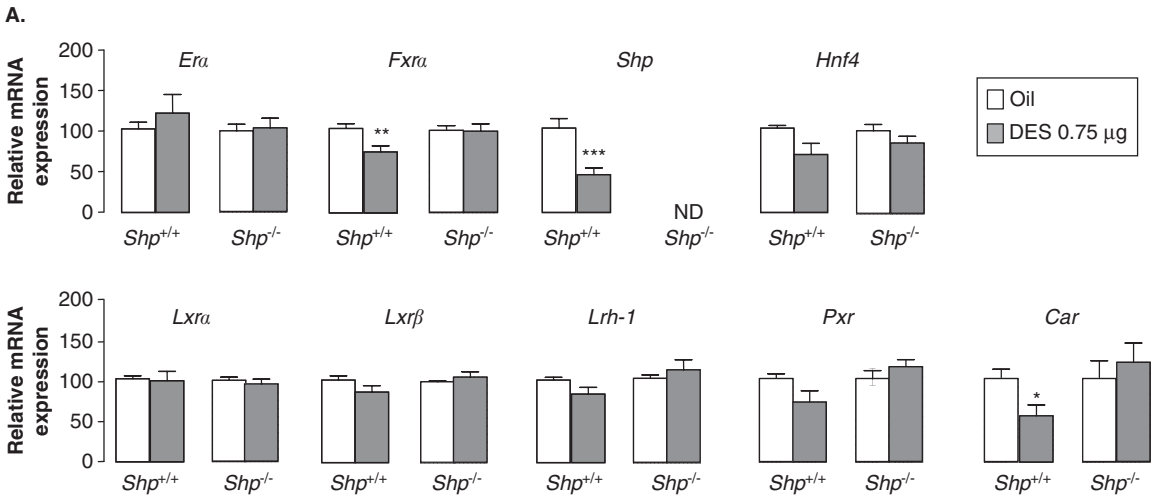

Figure 4. Neonatal exposure to DES alters mRNA accumulation of several nuclear receptor involved in liver physiology. mRNA expression of *Erα, Fxrα, Shp, Hnf4, Lxrα, Lxrβ, Lrh-1, Pxr,* and *Car* normalized to β-*actin* levels in liver of adult $Shp^{+/+}$ and $Shp^{-/-}$ mice exposed neonatally to vehicle, 0.75 µg or 5 µg of DES. In all of the panels data are expressed as the means ± SEM. Statistical analysis: *p < 0.05 versus control diet group; n = 6 – 10 per group.
DES: Diethylstilbestrol.

upon DES exposure, whereas no effect was observed in the $Shp^{-/-}$ mice (**Figure 3B**).

3.4 Neonatal exposure to DES alters adult liver histology

To continue the phenotyping of the livers exposed to DES, we analyzed them at the histological level. Neonatal exposure of the $Shp^{+/+}$ and $Shp^{-/-}$ males to DES produced no significant effects on adult body weight even at the high dose of DES (Supplemental Figure 1A). DES exposure led to a significant increase in liver weight in the $Shp^{+/+}$, whereas no significant effect was observed in the $Shp^{-/-}$ males even at the higher dose of DES (Supplemental Figure 1A). In the 10-week-old adult $Shp^{+/+}$ mice that were neonatally exposed to DES, the livers exhibited morphological alterations including the infiltration of lymphocytes (**Figure 3C**) and the presence of fibrotic areas (**Figure 3C**). These findings were further supported by the increased expression of CK8, a marker of liver injury (**Figure 3D**). The same results were obtained for the higher 5 µg dose (**Figure 3D**). No effects on the $Shp^{-/-}$ mice were observed even at the 5 µg DES dose (**Figure 3C and D**).

The increase in liver weight could have resulted from several mechanisms. No effect of DES exposure was observed on the rates of apoptosis in either the $Shp^{+/+}$ or $Shp^{-/-}$ males (data not shown). In contrast, immunochemistry for Ki-67, a marker of the cell cycle, revealed that the livers of the $Shp^{+/+}$ DES-treated group exhibited a greater proliferation rate compared to the vehicle-treated animals (**Figure 3C**). The $Shp^{-/-}$ males exhibited reduced sensitivity to DES exposure because the proliferation rate was not increased in these animals (**Figure 3E**).

3.5 The expressions of several NRs involved liver physiology are sensitive to neonatal exposure to DES

To define the altered signaling pathways that were potentially involved in the effects on DES on the liver, we studied the expressions of different NRs that are known to play critical roles in this process. In the wild-type males, no effects were observed on the mRNA accumulations of *Er-α*, *Lrh-1*, *Lxrα* or *Lxrβ*. In contrast, the mRNA accumulations of *Hepatocyte Nuclear Factor-4* (*Hnf4)*, *Shp,* and *Fxrα* were significantly decreased in DES-treated groups (**Figure 4A**). Additionally, the expressions of *Car* and *Pxr*, which are two NRs that are responsive to DES, were found to be decreased in the $Shp^{+/+}$ males. No effects were observed in the $Shp^{-/-}$ males.

3.6 The expressions of epigenetic factors are sensitive to neonatal exposure to DES

It could be hypothesized that the altered liver metabolism in the early postnatal age are responsible for the alterations in adult. However, the low sensitivity of the $Shp^{-/-}$ males to DES-treatment during postnatal development does not favor of this hypothesis. Indeed, the effects of 5 µg DES on the expressions of *Cyp7a1*, *Cyp7b1*, and *Cyp8b1* in the 10dpn $Shp^{-/-}$ males were not associated with altered BA levels in the adults. These findings suggest that other SHP-dependent mechanisms must be involved. As epigenetic mechanisms have been demonstrated to be key factors in the long-term effects of EDs [27], we analyzed critical factors in epigenetic

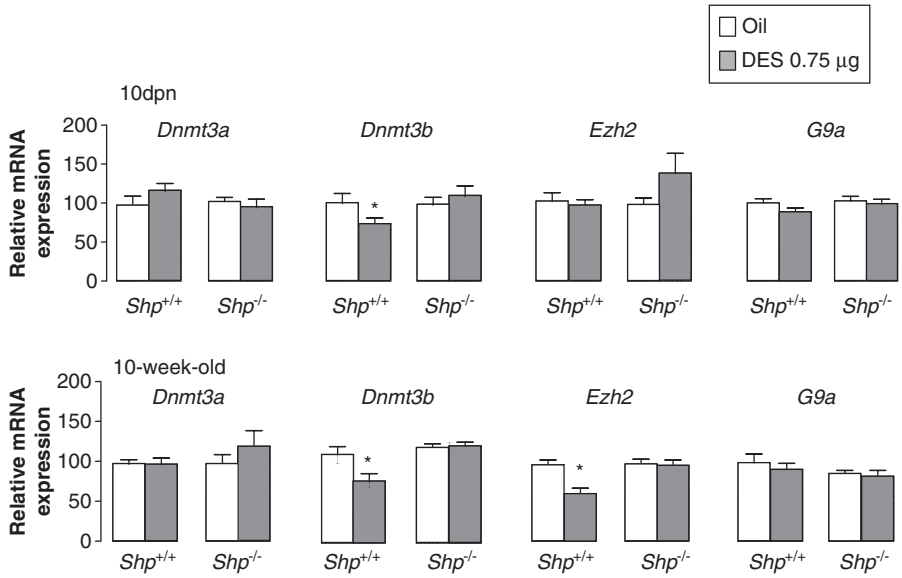

Figure 5. Neonatal exposure to DES alters mRNA accumulation of epigenetic factors. (A) mRNA expression of *Dnmt3a, Dnmt3b, Ezh2, G9a* normalized to β-*actin* levels in liver of 10 dpn pups or 10-week-old $Shp^{+/+}$ and $Shp^{-/-}$ mice exposed neonatally to vehicle or 0.75 µg of DES. In all of the panels data are expressed as the means ± SEM. Statistical analysis: *p < 0.05 versus control diet group; n = 6 – 10 per group.
DES: Diethylstilbestrol.

processes. The expressions of *Euchromatic Histone-lysine N-MethylTransferase 2* (*Ehmt2; G9a*), *DNA methyltransferase-1* (*Dnmt1*), *Dnmt2*, and *Dnmt3a* were not affected by DES exposure either at 10-days postnatal (dpn) or in adulthood (**Figure 5**). Regarding histone modifications, the accumulation of *Enhancer of Zeste homolog-2* (*Ezh2*) mRNA was decreased in the adult livers of the $Shp^{+/+}$ males, whereas no effect was observed at 10 dpn (**Figure 5**). Additionally, *Dnmt3b* mRNA accumulation was found to be decreased in the DES-treated $Shp^{+/+}$ males at both of the ages studied. No effects were observed in the $Shp^{-/-}$ mice (**Figure 5**). However, no modifications of global epigenetic markers were observed in the DES-treated animals (data not shown) suggesting additionally gene-specific alterations.

4. Discussion

Previous studies have reported the causal links between steroid hormones (androgens and estrogens) and liver diseases such as hepatocarcinoma [28] and cholestasis [29]. These relationships suggest that steroid pathways might play important roles in liver pathophysiology in the context of exposure to EDs.

The present study showed that neonatal exposure to an estrogenic compound altered the expressions of the genes involved in BA synthesis. Neonatal exposure to DES was associated with decreases in *Cyp7a1* and *Cyp8a1* expression, whereas *Cyp7b1* expression was increased. Interestingly, these effects were partly dependent on SHP because the as $Shp^{-/-}$ males were sensitive to the higher dose of DES. These results are consistent with those of previous study that showed that the effects of DES treatment in early postnatal life on the testes are SHP-independent [11]. These data suggest that SHP might not be a critical factor in liver and testes physiology in the context of estrogen exposure during early postnatal development.

In the adults, the involvement of SHP was supported by the lack of effect of DES exposure on the $Shp^{-/-}$ males. In the $Shp^{+/+}$ males, the BA metabolism was altered by DES-treatment through an effect on the BA synthesis pathway and on the catabolism and efflux of the BAs. The altered liver secretion of BAs was supported by the fact that the serum BA levels were not altered (data not shown). The significance of BA-elevation in liver was supported by the altered TG metabolism in response to neonatal exposure to DES.

Regarding the molecular signaling pathways, we demonstrate that, in the $Shp^{+/+}$ males, DES affected the expressions of *Shp, Fxrα,* and *Hnf4*, which are important regulators of liver physiology [4,30]. Additionally, DES led to reduced mRNA accumulations of the Constitutive Androstane Receptor (CAR, NR1I3) and the Pregnane X Receptor (PXR; NR1I2) in the $Shp^{+/+}$ males, whereas no effects were observed in the $Shp^{-/-}$ mice. These findings are consistent with the effect of DES on *Cyp3a4* and *Cyp2b10*, which are two target genes of *CAR or PXR* [31,32].

It could be hypothesized that the altered liver metabolism in the early postnatal age is responsible of the alterations observed in adulthood. However, the sensitivity of the $Shp^{-/-}$ males to DES-treatment during postnatal development does not favor this hypothesis. Thus, to establish the link between neonatal

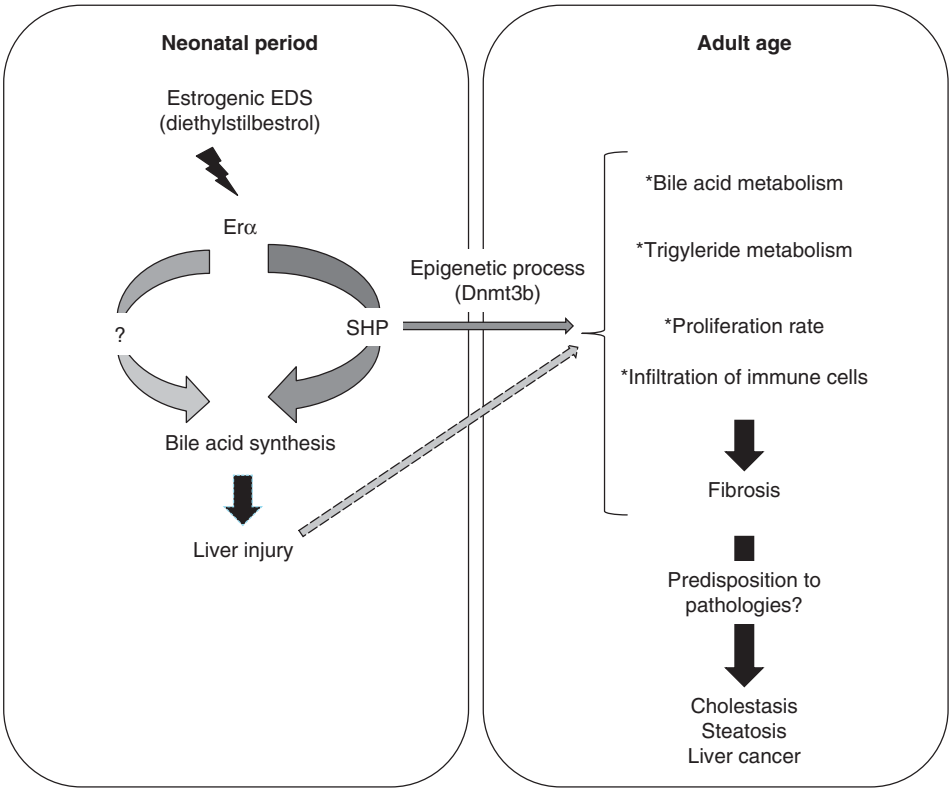

Figure 6. Proposed model. Estrogen-like EDs act through the activation of ERα altering genes involved in BA synthesis which in turn induced hepatotoxicity. Here we demonstrate that in neonatal age, these effects are SHP partly dependent suggesting SHP-independent mechanisms (left part). Interestingly, the neonatal exposure is associated with impacts on adult liver physiology as shown by altered BA and TG metabolisms as well as liver histology and proliferation rate. However, it could be assume that these phenomena in adult are not due to the alteration of BA metabolism in neonatal life, as there is a clear difference in the involvement of SHP at these different ages. This suggests that other mechanisms, may be through epigenetic factors, could be involved.

BA: Bile acids; ED: Endocrine disrupter; SHP: Small heterodimer partner; TG: Triglyceride.

exposure and permanent alterations in liver physiology following DES exposure, we analyzed epigenetic factors. Indeed, epigenetic mechanisms have been demonstrated to be key factors of the long-term effects of EDs [27]. Moreover, key metabolic genes involved in glucose and lipid metabolism are controlled by epigenetic processes [33,34]. Similarly, some of the epigenetic genes involved in histone modifications and DNA methylation were found to be altered following DES-exposure. Among these genes, it has previously been demonstrated that the levels of *Dnmt3b* are decreased in the seminal vesicles of animals exposed to DES [35]. Interestingly it has been suggested that alterations of *Dnmt3b* can modify liver metabolism [36]. To decipher the molecular mechanisms involved in this process, additional ChiP-seq experiments will be necessary to define the specific target genes that are altered by the down-regulation of Dnmt3b in the context of DES exposure.

The etiologies of liver pathologies remain difficult to establish. The significance of the effects of early postnatal exposures on later adult outcomes of liver disease is supported by the fact that the onsets of liver diseases are insidious, and there are often latent periods between the occurrence of the disease and its detection due to hepatic decompensation. Our results are interesting regarding the hypothesis of the developmental origin of health and diseases. Thus, the present work demonstrates that the history of exposure to EDs during early life needs to be taken into consideration as it affects adult liver physiology.

The present study supports this later effect of estrogenic exposure on liver physiology as shown by the increases in liver weight and CK8 protein accumulation following DES exposure. Interestingly, CK8 is a marker of liver injury [37,38]. Additionally, the overexpression of CK8 has been described in hepatocarcinomas in mice and humans [39,40]. Consistent with this potential pro-carcinogenic property of CK8, we showed that the livers of the mice that were exposed to DES exhibited increased proliferation rates, which could predispose them to cancer development at older ages.

This work leads us to propose a model for the actions of estrogen-like ED on liver physiology at different ages (**Figure 6**). As demonstrated previously for estrogens [19], estrogen-like ED might act through the activation of ERα

to alter genes involved in BA synthesis, which in turn induces hepatotoxicity. Here, we demonstrated that, at the neonatal stage and at the lower dose, these effects were partially dependent on SHP (**Figure 6**, left part). Interestingly, the neonatal exposure was associated with the effects on the liver physiology as shown by the SHP-dependent alterations in BA and TG metabolism and liver histology and proliferation rate. However, we assume that these phenomena in the adult are not completely determined by alterations of BA metabolism during neonatal life, as there were clear differences in the involvement of SHP at these different ages. Indeed, SHP is a critical factor during adulthood (**Figure 6**, right part), whereas this is less the case in early postnatal life.

Taken together, these data demonstrate that exposure to estrogenic EDs during the neonatal period alters liver physiology and could open a new field of research regarding the developmental origin of adult liver diseases, such as steatosis, cholestasis, and cancer. Moreover, we defined the role of SHP as a critical factor in the link between neonatal estrogenic signaling pathways and subsequent liver pathophysiology in adulthood.

Declaration of interest

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Supplementary materials available online

Supplemental Table 1 and Figure 1A.

Identification of the link between the hypothalamo-pituitary axis and the testicular orphan nuclear receptor NR0B2 in adult male mice.

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Endocrinology, 2014

Identification of the link between the hypothalamo-pituitary axis and the testicular orphan nuclear receptor NR0B2 in adult male mice

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The small heterodimer partner (NR0B2) is an atypical nuclear receptor known mainly for its role in bile acid homeostasis in the enterohepatic tract. We previously showed that NR0B2 controls testicular functions such as testosterone synthesis. Moreover, NR0B2 mediates the deleterious testicular effects of estrogenic endocrine disruptors leading to infertility. The endocrine homeostasis is essential for health as it controls many physiological functions. This is supported by a large number of studies demonstrating that alterations of steroid activity lead to several kinds of diseases such as obesity and infertility. Within the testis, the functions of the Leydig cells are mainly controlled by the hypothalamo-pituitary (HP) axis *via* luteinizing hormone/chorionic gonadotropin (LH/CG). Here we show that LH/CG represses *Nr0b2* expression through the PKA-AMPK pathway. Moreover, using a transgenic mouse model invalidated for *Nr0b2*, we point out that NR0B2 mediates the repression of testosterone synthesis and subsequent germ cell apoptosis induced by exposure to anti-GnRH compound. Together, our data demonstrate a new link between HP axis and NR0B2 in testicular androgen metabolism, making NR0B2 a major actor of testicular physiology in case of alteration of LH/CG levels.

Within the testis, Leydig cells ensure the synthesis of various hormones. Androgens are involved in the development of secondary sexual characters during puberty and the maintenance of fertility in the adulthood (1). Testosterone controls germ cell proliferation and survival (2, 3). Steroidogenesis is a multistep process. First, cholesterol is transported through the mitochondrial membrane by steroidogenic acute regulatory protein (StAR) (4) or peripheral benzodiazepine receptor (5). Then, cholesterol side chain is cleaved by cytochrome P450–11A1 (CYP11A1) producing pregnenolone (6). Several subsequent enzymatic reactions involving 3β-hydroxy-steroid dehydrogenase (3βHSD) and cytochrome P450–17A1 (CYP17A1) allow the production of testosterone (7).

The control of the Leydig cell functions, including steroidogenesis, is predominantly mediated by the hypothalamo-pituitary-gonadal axis via luteinizing hormone (LH)/chorionic gonadotropin (LH/CG) depending on the considered species (7). This results in de novo protein synthesis such as StAR (8). Under acute effect of LH/CG, free cholesterol is transferred to the inner membrane of mitochondria. Chronic stimulation leads to the expression of *Cyp11a1*, *3βhsd* and *Cyp17a1* (9). Among multiple signaling pathways, LH/CG stimulates cAMP production, which in turn regulates expression of steroidogenic en-

Abbreviations:

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zymes in Leydig cells. cAMP acts through a variety of transcription factors, including steroidogenic factor-1 (SF-1; NR5A1) (10), dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (DAX-1; NR0B1) (11), liver X receptor-α (LXRα; NR1H3) (12, 13), CCAAT/enhancer-binding protein-β (C/EBPβ) (14), liver receptor homolog-1 (LRH-1; NR5A2) (15), Nur77 (Nerve Growth factor IB (NGFIB); NR4A1) (16), and cAMP Response Element-binding protein (CREB) (17).

In a classical negative feed-back loop, the steroids produced by the testis, namely testosterone and estrogens, inhibit the secretion of GnRH by the hypothalamus and then the production of gonadotropins (LH and FSH). Steroids also inhibit LH secretion directly at the pituitary levels.

The small heterodimer partner (NR0B2, SHP) is a member of the nuclear receptor superfamily (18). Functions of NR0B2 have been linked to its ability to repress the transcriptional activity of other nuclear receptors such as NR5A2 (19, 20). In addition to the liver, *Nr0b2* has been demonstrated to be expressed in the testis. From puberty *Nr0b2* is expressed in interstitial cells (21). It represses steroidogenesis by limiting the expression of *Star*, *Cyp11a1* and *3βhsd*. NR0B2 inhibits the expression of steroidogenic genes, on one hand by inhibiting the expression of the nuclear receptors *Nr5a1* and *Nr5a2*, and on other hand by directly repressing the transcriptional activity of NR5A2 at least on the promoter of *Star* and *Cyp11a1* genes (21). This was supported by the higher testosterone levels in $Nr0b2^{-/-}$ males compare to $Nr0b2^{+/+}$ mice (21). This effect is mainly due to the intrinsic testicular role of NR0B2, as $Nr0b2^{-/-}$ males have normal plasma LH levels and normally respond to LH/CG stimulation (21). However, NR0B2 mediates testicular deleterious effects of estrogenic endocrine disrupters such as diethylstilbestrol (DES) or estradiol benzoate (EB) (22). An interesting point is the lack of effect of EB in $Nr0b2^{-/-}$ males as neonatal exposure to EB has been demonstrated to reduce plasma LH concentration (23). Combined, these data suggest that $Nr0b2^{-/-}$ Leydig cells should be resistant to the deleterious effect of low LH levels. Surprisingly, the links between NR0B2 and the LH/CG signaling pathways within Leydig cells have never been studied so far.

Here, we show that *Nr0b2* expression is regulated by the hypothalamo-pituitary (HP) axis. In vivo treatment with hCG results in the decrease of testicular *Nr0b2* mRNA accumulation. In vitro approaches, using the well-defined mouse Leydig MA10 cell line, demonstrate the involvement of PKA-AMPK signaling pathways. Consistently, we show that administration of an antagonist of GnRH-receptor (AG) induces an increase of *Nr0b2* mRNA accumulation leading to lower testosterone synthesis. This suggests a potential role of NR0B2 in the testicular impact of HP axis on testicular physiology. This was corroborated by the fact that *Nr0b2* deficiency protects male mice against the harmful effect on germ cell survival induced by an AG.

Materials and Methods

Animals. Mice used were previously described (21) and maintained on a mixed background (C57BL/6J/129sv), and were housed in controlled temperature rooms with 12h light/dark cycles, and had ad libitum access to food and water.

Mice were injected subcutaneously with hCG (5 IU, equivalent to 1.42 mM; Sigma-Aldrich, L'Isle D'Abeau, France) diluted in NaCl 0.09%. Regarding anti-GnRH (AG-045 572, Tocris, Bristol, United Kingdom) experiments, males were gavaged with 10 mg/kg during 12 hours or 3 days, or with vehicle (methylcellulose 1%, Sigma-Aldrich). This study was conducted in accordance with current regulations and standards approved by Institut National de la Santé et de la Recherche Médicale Animal Care Committee.

Histology. Testes from 10-week-old mice were collected, formalin-fixed and embedded in paraffin, and 5 μm-thick sections were prepared and stained with hematoxylin/eosin (n = 5–10 animals per group).

TUNEL analysis. TUNEL experiments were performed as previously described (24) on 5-μm of testis fixed in PFA 4%. In each testis, at least 100 random seminiferous tubules were counted. Results are expressed as the number of TUNEL-positive cells per 100 seminiferous tubules.

Endocrine investigations. Testosterone concentration was measured on heparin-treated plasma. Intratesticular testosterone was extracted as previously described (27). A commercial kit (Assay Designs, Ann Arbor, USA) was used for the assays.

Real-Time RT-PCR. RNA from testis samples were isolated using trizol® (Invitrogen Corporation, Carlsbad, CA). cDNA was synthesized from total RNA with the SuperScript II First-Strand Synthesis System (Life Technologies, Saint Aubin, France) and random hexamer primers. Real-time PCR measurement of individual cDNAs was performed using SYBR green dye to measure duplex DNA formation with the Eppendorf Realplex system. Sequences of the primers were given in previous studies: *Actin*, *Star*, *Cyp11a1* (13), *36b4* (12), *Nr0b2*, *Lrh-1* and *Sf-1* (25). Standard curves were generated with pools of testis cDNA from animals with different genotypes and/or treatments. Results were analyzed using the $\Delta\Delta$ct method.

Cell culture experiments. MA10 cells were previously used (26) and were maintained at 37°C in an atmosphere of 5% CO_2 with Waymouth (Life Technologies) containing 100 U/ml penicillin and 100 μg/ml streptomycin supplemented with 10% horse serum. On d0, MA10 cells were seeded at 400×10^3 cells

per well in 6-well plates and allowed to adhere overnight. The following day, cells were washed twice with 1× PBS, and medium without serum was applied with various ligands or vehicles. For hCG experiments vehicle was NaCl 0.09%, for Fsk (Sigma-Aldrich), 8BrcAMP (Sigma-Aldrich), PKI (R&D system), H89 (Sigma Adrich), PD98095 (R&D system) vehicle was DMSO (Sigma-Aldrich, 1/1000). Cells were harvested for mRNA or protein extractions.

Transient transfections. Mouse and human *Nr0b2* promoter luciferase reporter plasmids have been previously described (27). Briefly, *Nr0b2* regulatory sequences were cloned in PGL3 plasmid and correspond to sequence of −601 and +1 base pairs (bp) or −366 to +1 bp from the transcription initiation site. MA10 cells were transfected with lipofectamin (Invitrogen) in 24-well plates. *Nr0b2* promoter luciferase reporter construct (90 ng) was transfected together with expression plasmid encoding for β-galactosidase (90 ng). The quantity of DNA was maintained constant by addition of empty pCMX vector to a total amount of 240 ng of DNA per well. 24 hours after transfection, cells were treated with vehicle, hCG or Fsk. Cells were harvested 4 hours later and assayed for luciferase and β-galactosidase activities. Luciferase values were normalized to β-galactosidase activity.

Western blot analysis. Protein extracts (30 μg) from whole testis or MA10 cells were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Orsay, France). Membranes were incubated overnight at 4°C with primary polyclonal antibodies raised against AMPK (Cell Signaling #2532), P-AMPK (Cell Signaling #2531), ACC (Cell Signaling #3662), P-ACC (Ser79) (Cell Signaling #3661) or TUBULIN (1:30 000; from Sigma-Aldrich) (**see antibody Table 1**) followed by a 1-hour incubation with peroxidase-conjugated antirabbit or antimouse IgG (1:10000; Sigma-Aldrich). Peroxidase activity was detected using the Western Light System (PerkinElmer Life Sciences, Courtaboeuf, France).

Statistical Analysis. Two-way analysis of variance (ANOVA) was performed. When significant effects of treatment or genotype or their interactions were obtained, multiple comparisons were made with Turkey's test. All numerical data are represented as mean ± SEM. Significant difference was set at $P < .05$.

Results

hCG decreases Nr0b2 expression. To define in vivo the potential link between *Nr0b2* and LH/CG signaling pathway, we first analyzed the impact of LH/CG on *Nr0b2* mRNA expression. 12 hours (hrs) treatment with 5 IU of hCG resulted in a significant decrease in *Nr0b2* mRNA accumulation (**Figure 1A**). In the same line of evidence, treatment of male mice with a GnRH-receptor antagonist (AG-045 572; AG) induced *Nr0b2* mRNA accumulation (**Figure 1B & Suppl. 1A**). Note that *Star* mRNA accumulations mirror those obtained for *Nr0b2* (**Figure 1A & 1B**). To test whether LH/CG directly induces these effects on *Nr0b2* within the Leydig cells, we performed in vitro analyses using the well characterized mouse MA10 Leydig cell line. *Nr0b2* was decreased following hCG exposure since a dose of 0.025ng; the highest effect was observed at 25 nM (**Figure 1C**). The effect of hCG on *Nr0b2* accumulation was observed since 2 hours up to 12 hours after treatment (**Figure 1D**). As expected *Star* mRNA accumulations mirror those obtained for *Nr0b2* (**Figure 1C & 1D**).

Nr0b2 ***repression by hCG involves cAMP-PKA signaling pathway.*** In order to decipher the downstream signaling pathways induced by the LH-CG receptor, we used the adenylate cyclase activator, Forskolin (Fsk) in in vitro experiment using MA10 Leydig cell line. Results show that Fsk induced the repression of *Nr0b2* accumulation since 2.5 μM and after 4hrs of treatment (**Figure 2A & 2B**). As expected *Star* mRNA accumulation was increased following exposure to Fsk (**Figure 2A & 2B**).

The involvement of cAMP pathway was confirmed using 8-BromocAMP (8BrcAMP) (**Figure 3A**). Treatment for 4 hrs with 8BrcAMP resulted in *Nr0b2* repression and induction of *Star* mRNA accumulation (**Figure 3A**). In Leydig cells, PKA pathways play an important role in the control of steroidogenesis (28). To define if PKA was involved in *Nr0b2* regulation by cAMP, we used PKI and H89, two pharmacological inhibitors of PKA. Pretreatment with PKI or H89 abolished the effect of hCG on *Nr0b2* mRNA accumulation (**Figure 3B & 3C**). The efficiency of PKI or H89 treatments were supported by their impacts on hCG induced *Star* expression (**Figure 3B & 3C**). This is also highlighted by the induction of CREB phosphorylation by hCG which was minored by PKI and H89 exposures (**Figure 3D**). The role of PKA was confirmed, using PKI or H89, by the measurement of steroids (**Figure 3E**). In contrast, the use of PD98059, an inhibitor

Table 1. Antibody

Peptide/protein target	Antigen sequence (if known)	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
Phospho-ACC			Cell Signalling, #3661	Rabbit	1/2000
ACC			Cell Signalling, #23 662	Rabbit	1/2000
Phospha-AMPK			Cell Signalling, #2531	Rabbit	1/2000
AMPK			Cell Signalling, #2532	Rabbit	1/2000
TUBULIN			Sigma Aldrich,	Mouse	1/30 000

of ERK pathways, has only a slight impact on *Nr0b2* and *Star* expression (**Suppl. 1B & C**).

hCG signaling pathways act on* Nr0b2 *regulatory sequences. In order to show a direct impact of LH/CG signaling pathways on *Nr0b2* regulating sequences, various luciferase constructs of either mouse or human region of the *Nr0b2* promoter were used (20). hCG or Fsk treatments significantly repress luciferase activity using the –601;+1bp-luc construct, whereas the short construct (-333;+1pb) was not responsive to hCG or Fsk (**Figure 4**). Same results were obtained using the human *NR0B2* promoter (**data not shown**).

AMPK signaling pathways repress* Nr0b2 *expression in response to hCG. Recently, AMPK signaling pathway was demonstrated to play some role in the regulation of Leydig steroidogenesis (29). The functionality of AMPK signaling pathway in MA10 cells was tested using pharmalogical approach with its activator AICAR and the C-compound inhibitor (C-cpd). AICAR exposure increased the level of AMPK phosphorylation (P-AMPK) and of its target P-ACC (29) (**Figure 5A & 5B, left**). In contrast, the use of C-cpd counteracted the effect of AICAR on P-AMPK and P-ACC (**Figure 5A & 5B, right**). As AMPK pathway is a known inducer of *Nr0b2* expression in liver (30), the potential involvement of these pathways in MA10 cells was then tested. Treatment of MA10 cells with AICAR led to an increase of *Nr0b2* mRNA accumulation (**Figure 5C**). Consistently, pretreatment with C-compound abolished the effect of AICAR on *Nr0b2* mRNA accumulation (**Figure 5C**).

The link between AMPK pathway and hCG response was then analyzed. Pretreatment of cells with AICAR abolished the effect of hCG on *Nr0b2* mRNA accumulation (**Figure 5D**). hCG exposure led to decreased phosphorylation of AMPK (**Figure 5E & 5F, left**). The impact of this modification was supported by the lower phosphorylation of ACC, a known target of AMPK (**Figure 5E & 5F, left**). The pretreatment with AICAR counteract the impact of hCG on P-AMPK and then P-ACC (**Figure 5E & 5F, right**). In vitro, the impact of hCG on AMPK and ACC was confirmed using FSK on MA10 cell line (**Suppl. 1D**). In the same line of evidence, 8BrcAMP led to a decrease of AMPK and ACC phosphorylations (**Suppl. 1E**). The role of PKA was supported by the fact that PKI counteracted effect of hCG on P-AMPK and P-ACC (**Suppl. 1F**).

In addition, in vivo exposure of $Nr0b2^{+/+}$ mice to hCG reduced P-AMPK (**Figure 5G & 5H**). This was correlated with the decrease of the levels of P-ACC (**Figure 5G & 5H**). These data show that, in vivo, hCG exposure led to a decrease of P-AMPK as observed in MA-10 Leydig cell line. It is thus tempting to speculate that AMPK activation could regulate *Nr0b2* expression in response to LH/CG within the Leydig cells. However, it is important to

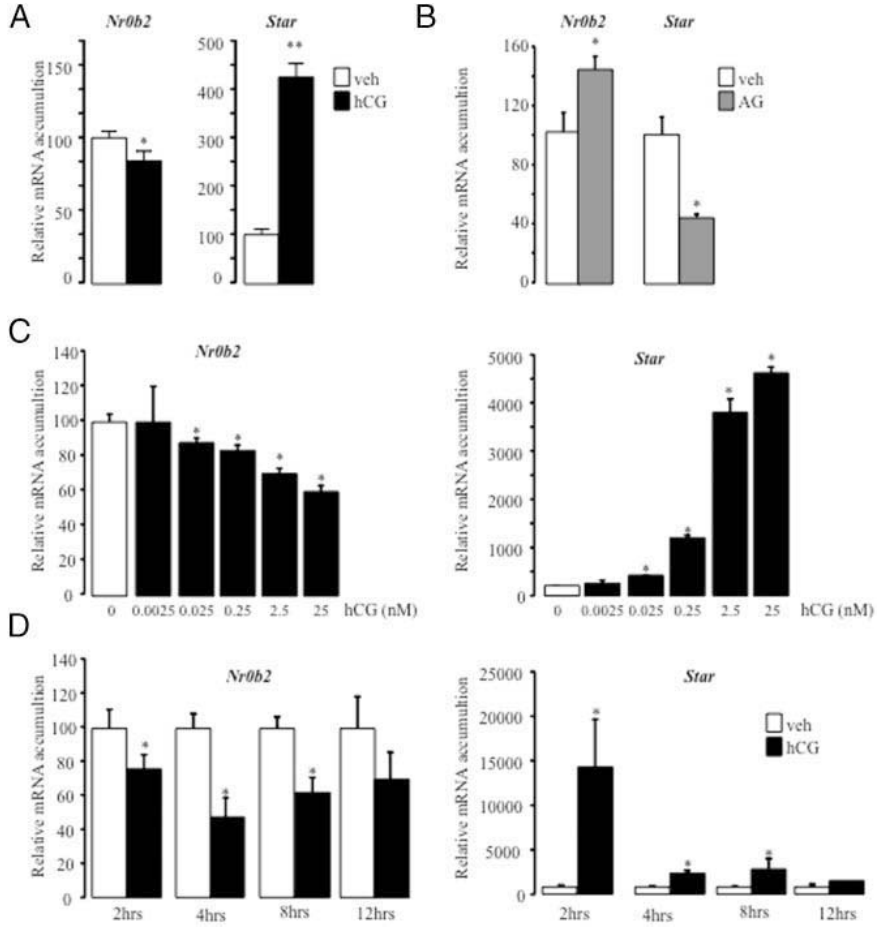

Figure 1. hCG signaling represses *Nr0b2* expression. **(A)** Testicular mRNA accumulation of *Nr0b2* and *Star* normalized to *Actin* mRNA levels in whole testes of C57BL/6J treated with vehicle or 5 IU hCG for 12 hours. **(B)** Testicular mRNA accumulation of *Nr0b2* and *Star* normalized to *Actin* mRNA levels in whole testes of C57BL/6J treated with vehicle (methylcellulose 1%) or 10 mg/kg of anti-GnRH (AG) for 12 hours. **(C)** mRNA expression of *Nr0b2* and *Star* normalized to *36b4* levels in MA10 Leydig cell line exposed to vehicle or hCG (from 0.0025 to 25 nM) over 4 hrs. (n = 12 per group). **(D)** mRNA expression of *Nr0b2* and *Star* normalized to *36b4* levels in MA10 Leydig cells exposed to vehicle or hCG (2.5 nM) for 2, 4, 8 or 12 hrs. (n = 12 per group). In all of the panels, data are expressed as the means ± SEM. Statistical analysis: *, $P < .05$; **, $P < .01$.

note that AMPK and its targets have also been described in several cell types within the testis including Sertoli and germ cells (31).

Nr0b2 ***deficiency protects mice from germ cell loss induced by an anti-GnRH.*** As previously demonstrated (21), *Nr0b2*$^{-/-}$ males presented higher intra-testicular testosterone levels (**Suppl. 2A**). To analyze the impact of NR0B2 in response to the modulation of HP axis, 10 week old adult *Nr0b2*$^{+/+}$ or *Nr0b2*$^{-/-}$ male mice were treated with a GnRH receptor antagonist (AG). Interestingly, 12 hours of treatment with AG led to a decreased intra-testicular levels of testosterone in *Nr0b2*$^{+/+}$ mice while no effect was observed in the *Nr0b2*$^{-/-}$ males (**Figure 6A**). The effect of AG was mainly supported by the HP axis as AG did not decrease expression of *Nr0b2* in MA10 Leydig cell line (**Suppl. 2B**). The effect of AG on testosterone synthesis in wild-type males was transitory as after 3-days of treatment the intra-testicular levels of testosterone were normalized (**Suppl. 2C**). Consistent with decreased testosterone levels 12hrs after the treatment, a decrease in mRNA levels of *Lhcgr*, *Star* and *Cyp11a1* was observed in *Nr0b2*$^{+/+}$ males (**Figure 6B**), whereas no modification of these genes was seen in *Nr0b2*$^{-/-}$ males (**Figure 6B**). The expression of *Nr5a1* and *Nr5a2*, which are known inducers of steroidogenesis and targets of NR0B2 were not affected by AG (**Figure 6C**). Moreover, AG had no effect on the expression of the negative regulator of steroidogenesis *Nr0b1* (**Figure 6C**). These results suggest that the effect of NR0B2 might be through its ability of inhibiting NR5A2 and/or NR5A1 activities on the regulatory sequences of the steroidogenic genes as previously demonstrated (21). These results show that NR0B2 is a key local factor of Leydig homeostasis.

Consistent with the lower testosterone levels in *Nr0b2*$^{+/+}$ mice (**Figure 6A**), a significant decrease of seminal vesicle weight, an androgen-dependent organ, was observed at 3 days after the beginning of the AG-treatment (**Figure 6D**). No effect on vesicle seminals was observed in *Nr0b2*$^{-/-}$ males highlighting the critical role of NR0B2 (**Figure 6D**). Likewise, AG exposure induced a significant increase in apoptotic spermatogenic cells in the testis of *Nr0b2*$^{+/+}$ mice, as determined by TUNEL analysis (**Figure 6E and 6F**). Such impact was not observed in *Nr0b2*$^{-/-}$ males.

Discussion

Endocrine homeostasis is a critical physiological process as alteration could lead to various diseases. This point has been enlightened in the last decades with the large impact of endocrine disrupters on animal and human health (32, 33). Among the factors defined to play important roles in steroid homeostasis, NR0B2 has been demonstrated to control male sexual maturity through testicular physiology (21).

It has been previously proposed that NR0B2 might contribute to local regulation of steroidogenesis (21). Indeed, *Nr0b2*$^{-/-}$ males have higher testosterone levels. Moreover, in vitro experiments show that NR0B2 overaccumulation has been demonstrated to repress the expression of steroidogenic genes via NR5A1 and/or NR5A2. However, the testicular regulation of *Nr0b2* expression has not been explored so far.

Previous data led us to hypothesize that NR0B2 should be involved in the control of basal expression of steroidogenic genes. However, we point out that *Nr0b2* expression is increased in case of lower activity of HP axis leading to decreased LH/CG levels and repressing testosterone synthesis. At the molecular level, we show that *Nr0b2* expression is controlled within the Leydig cells by cAMP/PKA/AMPK pathway after LH/CG exposure. The link between NR0B2 and LH/CG signaling was

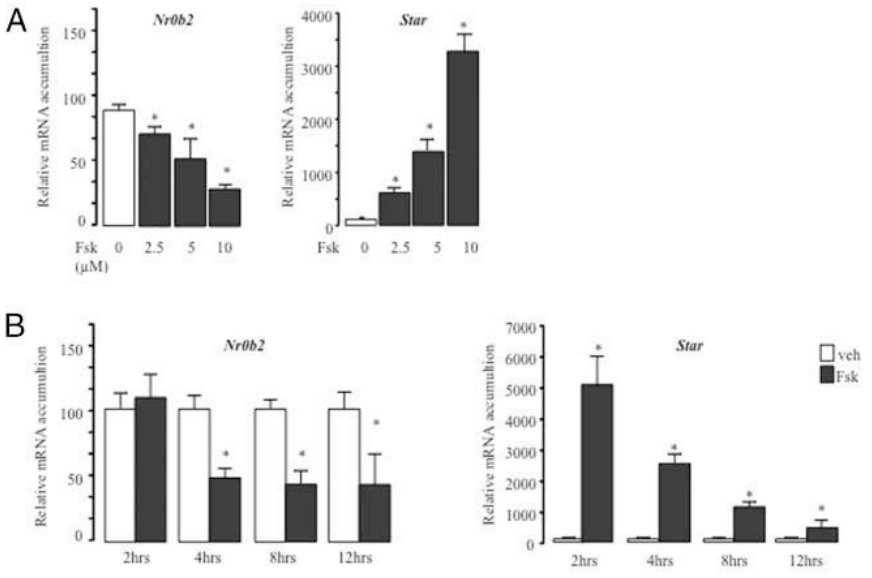

Figure 2. Adenylate cyclase activator, Fsk, represses *Nr0b2* expression. **(A)** mRNA expression of *Nr0b2* and *Star* normalized to *36b4* levels in MA10 Leydig cells exposed to vehicle or Fsk (from 2.5 to 10 μM) over 4 hrs, (n = 12 per group). **(B)** mRNA expression of *Nr0b2* and *Star* normalized to *36b4* levels in MA10 Leydig cell line exposed to vehicle or Fsk (5 μM) for 2, 4, 8 or 12 hrs, (n = 12 per group). *, $P < .05$; **, $P < .01$.

demonstrated here using an inhibitor of the HP axis (AG). This effect was associated with an increased apoptosis of germ cells following AG exposure. This involvement of NR0B2 is supported by the fact that *Nr0b2*$^{-/-}$ males are protected from the deleterious effects induced by AG exposure. The presented results lead us to propose a new model regarding the role of NR0B2 in the control of the steroidogenesis that could be relevant in cases of altered HP axis function (**Figure 7**).

In a physiological situation, LH is pulsatly secreted by pituitary and interacts with its receptor on the Leydig cells. This will increase the intracellular concentration of cAMP and activate PKA. Stimulated PKA will thus induce the production of steroids by the well-known pathway CREB/P-CREB, as shown in figure 7. Our data clearly point out a new regulatory pathway where NR0B2 plays an important role. Indeed, stimulated PKA will diminish AMPK activation which in turn results in the repression of *Nr0b2* expression. Finally, testosterone (T) and estradiol (E2) will have their physiological effects on target organs. Among them, T and E2 will exert a negative feedback by decreasing LH secretion. As expected, we show that during that feedback regulation, P-AMPK is increased followed by higher NR0B2 levels. As already described NR0B2 will thus inhibit NR5A1 and NR5A2 transcriptional effects on various steroidogenic genes such as *Star*.

Our data are in line with previous studies showing that the invalidation of AMPKα1 impacts testicular physiology. AMPK is expressed in several cell types of the testis such as Leydig, Sertoli and germ cells. In AMPK-/- mice, spermatozoa show defects of mitochondrial activity and morphological defects. In addition, these AMPK-/- males show a hyperandrogeny, associated with altered steroidogenesis. This is due to the increase of cholesterol content, the precursor of steroid synthesis, a higher mRNA accumulation of steroidogenic genes consistent with a higher level of P-CREB. These results clearly sustain our present study suggesting the impact of AMPK on steroidogenesis and its crosstalk with hCG/cAMP/CREB pathway. In addition, Abdou et al demonstrated that in MA10 cells, the

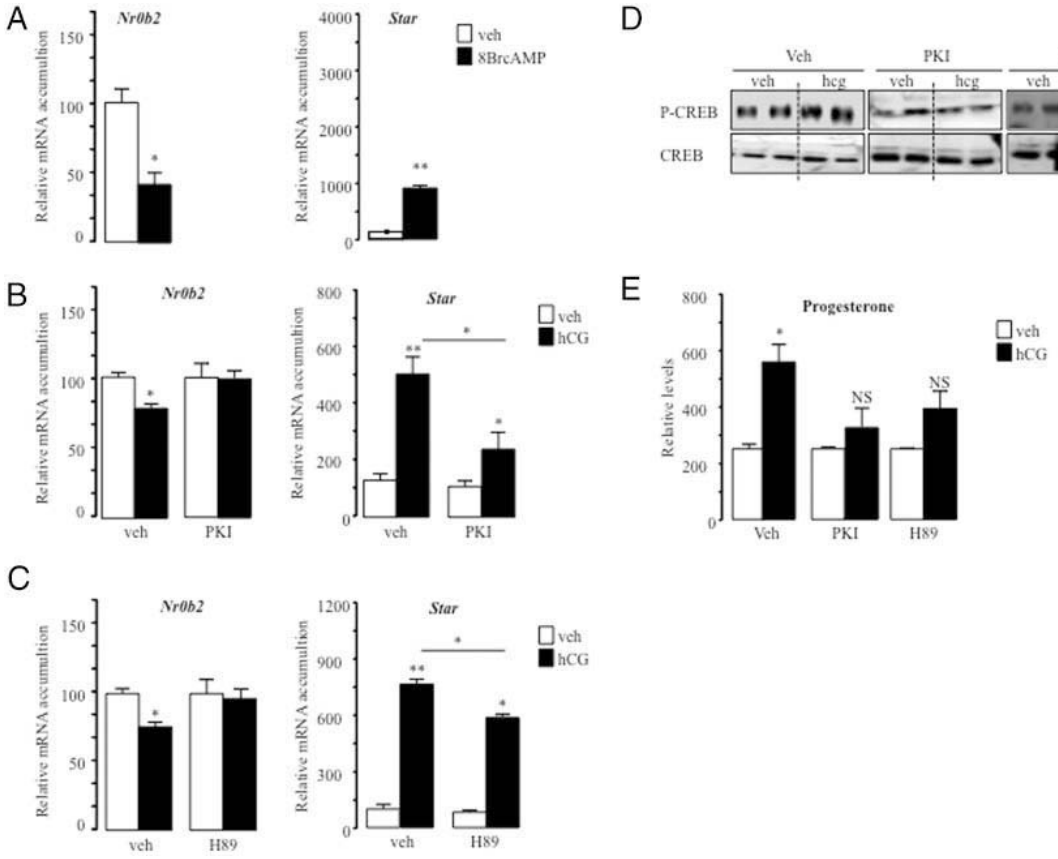

Figure 3. cAMP/PKA signaling controls *Nr0b2* expression in Leydig MA10 cell line. A, mRNA expression of *Nr0b2* and *Star* normalized to *36b4* levels in MA10 Leydig cell line exposed to vehicle or 8BrcAMP over 4 hrs, (n = 12 per group). **(B)** mRNA expression of *Nr0b2* and *Star* normalized to *36b4* levels in MA10 Leydig cells pretreated with PKI for 2 hours and then treated with vehicle or hCG (0.25 nM) for 4hrs, (n = 12 per group). **(C)** mRNA expression of *Nr0b2* and *Star* normalized to *36b4* levels in MA10 Leydig cells pretreated with H89 for 2 hours and then treated with vehicle or hCG (0.25 nM) for 4hrs, (n = 12 per group). **(D)** Immunoblots of CREB and Phospho-CREB in MA10 Leydig cell pretreated with PKI or H89 for 2 hours and then treated with vehicle or hCG (0.25 nM) for 4hrs, (n = 12 per group). **(E)** Relative levels of progesterone in medium of MA10 Leydig celsl pretreated with PKI or H89 for 2 hours and then treated with vehicle or hCG (0.25 nM) for 4hrs, (n = 4 to 6 per group). In all of the panels, data are expressed as the means ± SEM. Statistical analysis: *, $P < .05$; **, $P < .01$; ***, $P < .005$.

use of AICAR counteracts the effects of forskolin or hCG on steroid production and Star expression (34). These results are clearly those that we are also describing in our manuscript. Consistently, siRNA directed against AMPK led to higher sensitivity of MA10 to forskolin regarding steroid synthesis and Star expression. Previous studies (34, 29) also demonstrated that the effect of AMPK involved NR5A1/2 sites. Interestingly, as previously discussed in our manuscript, NR0B2 is known to repress these nuclear receptors. Altogether these results support our work on the role of NR0B2 in AMPK effects on steroidogenesis.

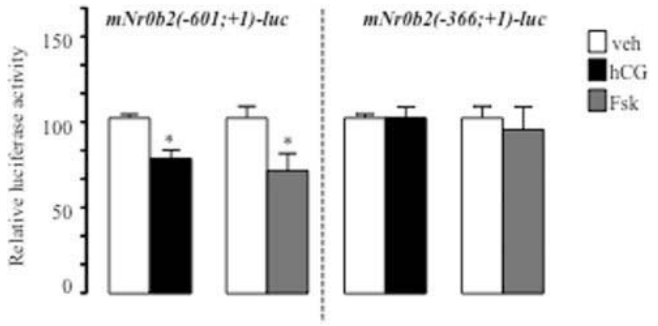

Figure 4. *Nr0b2* promoter is controled by hCG signaling. A, Transient transfection assay of MA10 cells transfected with a mouse *Nr0b2* promoter luciferase reporter plasmid and exposed to vehicle, hCG (2.5 nM) or Fsk (5 μM) over 4 hrs. Luciferase activity was normalized with β-gal and was expressed as relative light units (RLU) of triplicate assays (mean ± SD). *, $P < .05$.

LH signaling leads to cAMP production and then induction of steroidogenesis. Interestingly, it has been demonstrated that following hCG treatment, phosphor-diesterases (PDEs) transform cAMP into AMP which results in turn to AMPK activation. This induction of AMPK then represses steroidogenesis. This acts as a feedback mechanism to avoid maintenance of high steroid synthesis (Figure 7). In that context, it could be hypothesized that data using cAMP analogs should be analyzed with caution as they will act in favor to steroidogenesis activation and

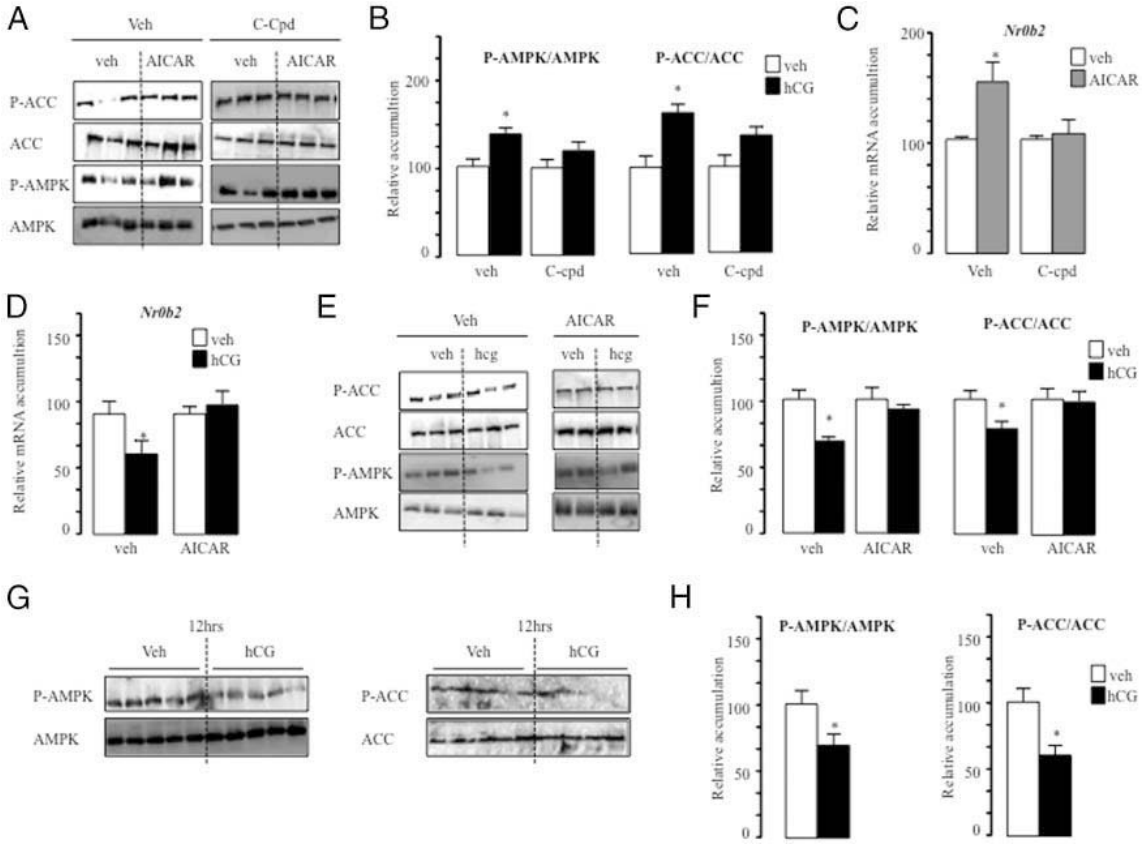

Figure 5. hCG signaling represses *Nr0b2* expression through AMPK inhibition. A, Protein accumulation of ACC, P-ACC, AMPK and P-AMPK in MA10 Leydig cells treated with AICAR for 4hrs in combination or not with C-cpd, (n = 12 per group). **(B)** Quantification of Phospho-ACC, Phospho-AMPK protein accumulations compared to ACC and AMPK respectively. **(C)** mRNA expression of *Nr0b2* normalized to *36b4* levels in MA10 Leydig cells treated with AICAR in combination or not with C-cpd) for 4hrs, (n = 12 per group). **(D)** mRNA expression of *Nr0b2* normalized to *36b4* levels in MA10 Leydig cells pretreated with AICAR for 2 hours and then treated with vehicle or hCG (2.5 nM) for 4hrs, (n = 12 per group). **(E)** Protein accumulation of ACC, Phospho-ACC, AMPK and Phospho-AMPK in MA10 Leydig cells pretreated with AICAR for 2 hours and then treated with vehicle or hCG (2.5 nM) for 4hrs, (n = 12 per group). **(F)** Quantification of Phospho-ACC Phospho-AMPK protein accumulations compared to ACC and AMPK respectively. **(G)** Immunoblots of cleaved ACC, Phospho-ACC, AMPK and Phospho-AMPK performed on testicular protein extracts of mice exposed 12 hrs to 5 IU of hCG, (n = 6–10 per group). **(H)** Quantification of Phospho-ACC, Phospho-AMPK protein accumulations compared to ACC and AMPK respectively (n = 6–10 per group). Vehicle treated mice were arbitrarily fixed at 100%. In all of the panels, data are expressed as the means ± SEM. Statistical analysis: *, $P < .05$; **, $P < .01$.

repression of AMPK activity. In that case it will be more difficult to visualize the kinetic event of the effects of PDE leading to production of AMP and then activation of AMPK.

Altogether, we hypothesize that the physiological role of NR0B2 might be to decrease the expression of steroidogenic genes in response to the central negative feedback on LH. This might participate to maintain the normal pulsatile secretion of GnRH and gonadotropins and in turn ensure normal testicular physiology.

Paradoxically, *Nr0b2*$^{-/-}$ males present normal level of LH and normal answer to acute LH surge (21). This normal response to LH could be due to the fact that NR0B1, a "twin" receptor of NR0B2, can overcome its absence. Indeed, NR0B1 is also involved in the regulation of steroidogenesis (7). This potential redundancy is supported by the fact that *Nr0b1* gene shows the same expression pattern than *Nr0b2* in response to hCG (35).

Increase of NR0B2 in condition of low LH might lead to a repression of steroidogenesis. In turn, lower testosterone might be followed by a decrease of negative feedback at hypothalamo-pituitary levels and then might increase the subsequent activity of the HP axis. Thus it could be speculated that in long term experiments, there could be a higher testosterone production in *Nr0b2*$^{-/-}$ mice compared to wild-type males. Such hypothesis has to be further studied. However, as discussed, the lack of NR0B2 associated with higher testosterone levels was not associated

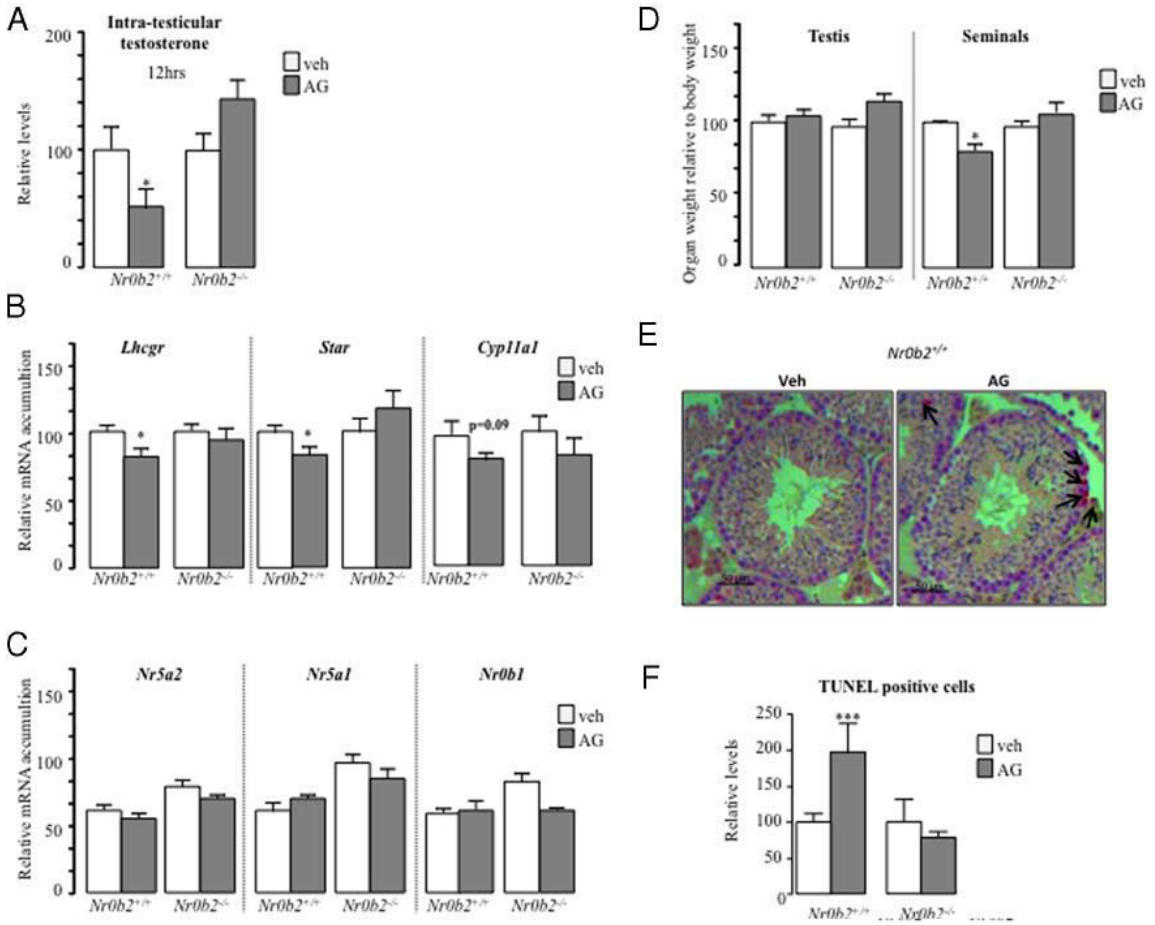

Figure 6. ***Nr0b2*** **deficiency protects testis from deleterious effect of an anti-GnRH exposure. A**, Relative intra-testicular testosterone concentration in *Nr0b2*$^{+/+}$ and *Nr0b2*$^{-/-}$ mice treated for 12hrs with vehicle of AG (10 mg/kg), (n = 5–10 per group). Vehicle treated mice of each genotype were arbitrarily fixed at 100%. **(B)** Testicular mRNA expression of *Lhcgr*, *Star* and *Cyp11a1* normalized *36b4* levels in whole testis of *Nr0b2*$^{+/+}$ and *Nr0b2*$^{-/-}$ mice exposed to AG (10 mg/kg/d), (n = 5 to 10 per group). Vehicle treated mice of each genotype were arbitrarily fixed at 100%. **(C)** Testicular mRNA expression of *Nr5a2*, *Nr5a1* and *Nr0b1* normalized to *36b4* levels in whole testis of *Nr0b2*$^{+/+}$ and *Nr0b2*$^{-/-}$ mice exposed to AG (10 mg/kg/d), (n = 5 to 10 per group). Vehicle treated mice were arbitrarily fixed at 100%. **(D)** Testis and seminal vesicle weights normalized to body weight in *Nr0b2*$^{+/+}$ and *Nr0b2*$^{-/-}$ mice exposed 3 days with vehicle of AG (10 mg/kg), (n = 5–10 per group). Vehicle treated mice of each genotype was arbitrarily fixed at 100%. **(E)** Apoptosis in *Nr0b2*$^{+/+}$ and *Nr0b2*$^{-/-}$ mice exposed to vehicle or AG, (n = 5–10 per group) analyzed by TUNEL staining. Representative micrographs of the testis of *Nr0b2*$^{+/+}$ or *Nr0b2*$^{-/-}$ exposed to vehicle or AG. The arrow-heads indicate apoptotic spermatocytes. The original magnification was x200. **(F)** Quantification of the TUNEL analyses. The number of TUNEL-positive spermatocytes is indicated as the number of positive cells per 100 seminiferous tubules, (n = 5–10). Vehicle treated mice of each genotype were arbitrarily fixed at 100%. In all of the panels, data are expressed as the means ± SEM. Statistical analysis: *, $P < .05$; **, $P < .01$; ***, $P < .005$.

with changes in the HP axis activity. This suggests that other factors might be involved. The lack of association between plasma testosterone and LH levels has been observed in several other studies (36, 37). In all these cases, estrogen levels should be measured as they are more potent inhibitors of the HP axis than testosterone. Altogether, these data confirm that the relation between the levels of sex steroids and HP axis is complicated. Our results also open a new field of investigation. Indeed, hypogonadotropic hypogonadism accounts for up to 2% of infertile men. Even though, it can be successfully treated (38), our results suggest that NR0B2 could be a new potential pharmacological target in such disease. Alterations of the HP axis could be due either to genetic mutation or exposure to endocrine disrupters (Figure 7). Indeed, if EB exposure has been associated with low LH/CG plasma levels and low testosterone synthesis (39), *Nr0b2*$^{-/-}$ males do not show reduced testosterone levels in response to estradiol benzoate exposure (22). This clearly suggests that NR0B2 might be a mediator of low LH signaling within Leydig cells in case of EED exposure.

Even though a full understanding of the mechanisms requires further studies, our work sustained the idea that AMPK might be an important actor of Leydig cell physiology as proposed by others (29). Moreover, we describe a critical role of NR0B2 in Leydig cell functions, and define it as a testicular messenger of the HP axis signaling in case of low LH levels.

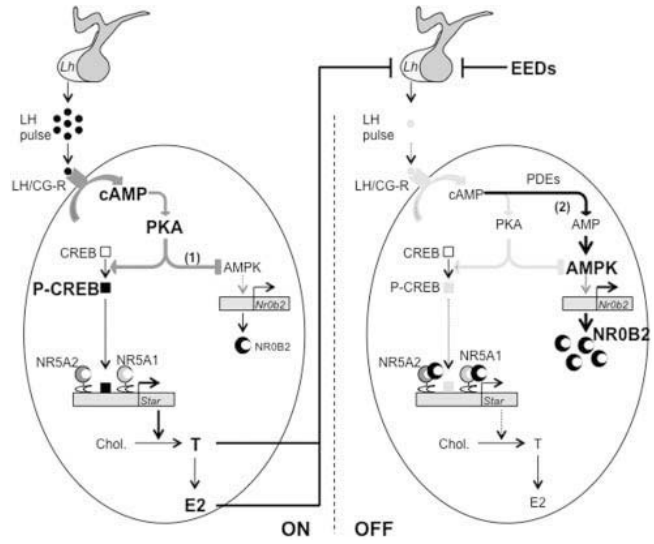

Figure 7. Role of NR0B2 in the control of testicular steroidogenesis, a proposed model. When LH is secreted, cAMP-PKA pathway is activated and the system is "ON". P-CREB, NR5A1 and NR5A1 will transcriptionnaly regulate *StAR* expression. At the same time, *Nr0b2* expression is maintained low via the inhibition of AMPK pathway. T and E2 increase will induce a negative feedback on LH secretion. Production of AMP by phospho-diesterases (PDEs) and decreased activity of cAMP-PKA pathway resulted in an increase of P-AMPK levels leading to NR0B2 accumulation, which in turn, through its interaction with NR5A1 and NR5A2, will reduce Star expression and steroid production. In pathophysiological condition, this low LH/CG levels could be observed in the context of estrogenic endocrine disrupters (EEDs) exposure. Indeed, exposure to EEDs results in a lower LH secretion and thus can induce testicular deleterious effects via the same signaling pathway involving NR0B2.

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Bile acid alters male mouse fertility in metabolic syndrome context.

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RESEARCH ARTICLE

Bile Acid Alters Male Mouse Fertility in Metabolic Syndrome Context

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Abstract

Bile acids have recently been demonstrated as molecules with endocrine activities controlling several physiological functions such as immunity and glucose homeostases. They act mainly through two receptors, the nuclear receptor Farnesol-X-Receptor alpha (FXRα) and the G-protein coupled receptor (TGR5). These recent studies have led to the idea that molecules derived from bile acids (BAs) and targeting their receptors must be good targets for treatment of metabolic diseases such as obesity or diabetes. Thus it might be important to decipher the potential long term impact of such treatment on different physiological functions. Indeed, BAs have recently been demonstrated to alter male fertility. Here we demonstrate that in mice with overweight induced by high fat diet, BA exposure leads to increased rate of male infertility. This is associated with the altered germ cell proliferation, default of testicular endocrine function and abnormalities in cell-cell interaction within the seminiferous epithelium. Even if the identification of the exact molecular mechanisms will need more studies, the present results suggest that both FXRα and TGR5 might be involved. We believed that this work is of particular interest regarding the potential consequences on future approaches for the treatment of metabolic diseases.

Introduction

Metabolic syndrome (MetS) has been linked with several abnormalities including overweight, dyslipidemia, hypertension and impaired glucose metabolism [1]. The numerous deleterious effects of MetS are being investigated throughout the medical community as MetS may affect many aspects of human physiology due to its systemic nature.

It has been proposed since 10 years that derivatives of bile acids (BAs) could be interesting molecules for the treatment of diseases of MetS such as diabetes or obesity. Indeed, BAs are being appreciated as complex metabolic integrators and signaling factors [2]. Through activation of diverse signaling pathways, BAs regulate their own synthesis, enterohepatic

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recirculation, as well as triglyceride, cholesterol, energy and glucose homeostases. BAs act mainly through two specific receptors, the nuclear receptor Farnesol-X-Receptor alpha (FXRα) and the G-protein coupled receptor TGR5 [3]. In order to develop promising novel drug targets to treat common hepatic and metabolic diseases, a lot of work has been done to better define the respective involvement of both receptors in different physiological pathways

This is a challenge as synthetic agonist for either FXRαor TGR5 could have either beneficial or deleterious impacts. Indeed, if FXRα agonists seem to be good candidate in treatment of diabetes [4], their utilization for the treatment of obesity can worsen the pathology [4]. Thus clear establishment of the involvement of each BA signaling pathways remain to be defined before using them as therapeutical drugs. Moreover, activation of such important signaling pathways could have some secondary effects on health.

In that line, our recent findings demonstrate the impact of BA on male fertility and testicular physiology. Indeed, BAs were shown to impact testicular physiology either via TGR5 or FXRα. TGR5 mainly acts in germ cell lineage. Its activation by BA represses network of cell-cell interactions through the downregulation of N-Cadherin as well as Cx43 expression through regulation of the transcriptional repressor, T-box transcription factor 2 gene [5]. This leads to germ cell sloughing and rupture of the blood-testis barrier and then apoptosis of spermatids. In parallel, FXRα has been demonstrated in several studies to control the testicular endocrine function supported by the Leydig cells *in vivo* [6]. Short term exposure to FXRα agonist (GW4064) represses testosterone synthesis. At the molecular level, FXRαactivation stimulates the expression of the small heterodimer partner (SHP) which in turn inhibits the expression of steroidogenic genes, on the one hand by inhibiting the expression of the nuclear receptors steroidogenic factor–1 (SF–1) and liver receptor homolog–1 (LRH–1), and on the other hand by directly repressing the transcriptional activity of LRH–1.

Regarding the links between BA signaling pathways and male testicular physiology and subsequent fertility disorders, we wondered what could be the consequences of a long term exposure to molecules that activate BA signaling pathways during the treatment of MetS diseases such as obesity. We thus aimed to define the equilibrium between potential positive or negative impacts of "BA treatment". We asked whether the BA-treatment will improve general heath of the mice and then fertility, or if BA will mainly have deleterious effects on fertility as we have demonstrated recently [5]. For that purpose, we decided to induce overweight in mice with high fat-diet (HFD) and then "treat" them with BAs to reverse the overweight as defined by Watanabe *et al.* [7].

Here we demonstrate that in mice fed a high fat diet, BA exposure leads to increased rate of male infertility. This is associated with alterations of testicular physiology.

Materials and Methods

Ethics Statement

This study was conducted in accordance with the current regulations and standards approved by the Animal Care Committee (CEMEA Auvergne; protocol CE 08–12).

Animals

C57Bl/6J were purchased from Charles River Laboratories (L'Arbresle, France). Mice were housed in temperature-controlled rooms with 12 hours light/dark cycles. Mice had *ad libitum* access to food and water. Five-week-old mice were exposed to D04-diet or 235HF diet (Control) for 3 months. Half of the group fed HFD were maintained either on 235HF diet or the 235HF supplemented with 0.5% cholic acid (HF-CA-diet) (SAFE, Augy, France) for 2 or 4 months to visualize the impact of BA exposure on overweight.

Fertility assement

Fifteen days before the sacrifice each male is put into reproduction during the day, without food (avoiding contamination of the females), with two unexposed C57Bl6J females (Charles River) (3 to 4 males per group per experiment). Breeding was daily monitored for the presence of a vaginal plug to determine whether mating occurred. Before the night, males were put back with specific diet (regarding the group). After 19–20 d, efficacy of mating was visually inspected by the female delivery; and the number of pups per litter was counted.

For analysis of blood testicular barrier (BTB) integrity, 15 μL of EZ-Link Sulfo-NHS-LC-Biotin (7.5 mg/mL; Thermo Fisher Scientific, Brebières, France) were injected into the left testis of anesthetized males exposed to a HFD or HF-CA diet [5]. Then, after 20 min., testes were removed, PFA-fixed and embedded in paraffin, and 5-μm-thick sections were prepared and stained for biotin.

Histology

TUNEL and Ki67 experiments were performed as reported in [8], [9]. We performed the cell counts in two to three independent experiments with at least 5 mice per group. In addition, for each male, counting was made on 2 non following slides.

Testis weight, the number of tubules per section and the evaluation of tubule diameter were not affected between groups. As all these parameters are equal for the different groups there might be no bias in the count of positive cells or tubes.

TUNEL analysis

Five-micrometer-thick paraffin-embedded sections were deparaffined with toluol followed by rehydratation. The slides of each group were incubated for 5 min in unmasking buffer (citrate acetate 1.8 mm, sodium citrate 8.2 mm, pH 6.0) at 86 C. Then the slides were incubated with 0.3 U/μl terminal deoxynucleotidyl transferase (Euromedex, Mundolsheim, France), 6.7 mm biotin-11-dUTP (Euromedex), and 26.7 mm dATP (Promega, Charbonnières, France) in terminal deoxynucleotidyl transferase buffer 1 h at 37 C. For the negative control, the enzyme was omitted. Extravidin alkaline phosphatase conjugate (dilution 1:100; Sigma-Aldrich) was added onto the slides for 25 min. Sigmafast FastRed TR/Naphthol AS-MX (Sigma-Aldrich) was used as the substrate according to the manufacturer's instructions. Counterstain was performed with Mayer's hematoxylin solution (Sigma-Aldrich) for 30 sec. In each testis, at least 100 random seminiferous tubules were counted. Results are expressed as the number of TUNEL-positive cells per 1000 seminiferous tubules. We perform the cell count (for TUNEL) in two to three independent experiments with at least 5 mice per group. In addition, for each male, counting was made on 2 non following slides.

Ki67 counting

Five-micrometer-thick paraffin-embedded sections were fixed 10 min in 4% paraformaldehyde and washed three times for 10 min in 1× PBS. Cells were permeabilized with 0.1% Triton X–100 and 0.1% citrate solution in PBS for 2 min at 4 C. Slides were incubated with anti Ki67 1/500 (Tebu-bio, Le Perray en Yvelines, France) overnight at 4 C and then washed three times in 1× PBS. Slides were incubated for 1 h at room temperature with a goat anti-rabbit secondary antibody labeled with Alexa 488 (1/250; from Invitrogen Detection Technologies, Cergy-Pontoise, France). In each testis, at least 100 random seminiferous tubules were counted. Results are expressed as the number of Ki67-positive cells per 100 seminiferous tubules.

Immunohistochemistry

Paraffin sections of PFA-fixed testis were sectioned at 5 μm. The sections were mounted on positively charged glass slides (Superfrost plus, Thermo Scientific, France), deparaffinized, rehydrated, treated for 20 min at 93–98°C in citric buffer (0.01 M, pH 6), rinsed in osmosed water (2 x 5 min) and washed (2 x 5 min) in Tris-buffered saline. Immunohistochemistry was conducted according to the manufacturer's recommendations, as described earlier [10]. Slides were then counterstained with Hoestch medium (1 mg/ml). The antibodies used are given in supplemental information (S1 Table).

Endocrine Investigations

Steroids were extracted from frozen testis (20 mg) with 10 vol of ethylacetate-isooctane (30:70, v:v) as previously described [9], and were measured using commercial kits: testosterone (Diagnostic Biochem, London, Canada).

Cholesterol, cholesterol esters and triglycerides were extracted from frozen testis as described previously with Folch method [11]. Cholesterol and cholesterol ester measurements were performed as recommended by manufacturer (Cholesterol and cholesterol ester: Calbiochem 428901; Triglycerides: Diagnostic Sys-tem, Holzheim Germany).

Glucose was measured on plasma upon manufacturer recommendations (Biomerieux 61269).

Real-Time RT-PCR

RNA from testis samples were isolated using Nucleospin RNA (Macherey-nagel, Hoerdt, France). cDNA were synthesized from total RNA with the MMLV reverse transcriptase and random hexamer primers (Promega, Charbonnière Les Bains, France). The real-time PCR measurement of individual cDNAs was performed using SYBR green dye (Master mix Plus for SYBR Assay, Eurogentec, Angers, France) to measure duplex DNA formation with the Eppendorf Realplex system. The sequences of primers are reported in S2 Table. Standard curves were generated with pools of testis cDNA from animals with different treatments. The results were analyzed using the ΔΔct method.

Western blot

Proteins were extracted from tissues using lysis buffer (0.4 M NaCl, 20 mM Hepes, 1.5 mM$MgCl_2$, 0.2 mM EDTA, 0.1% NP40, 1X protease inhibitors (Roche Diagnostics, Meylan, France)). Antibodies were used in TBS, 0.1% tween and 10% milk. The antibodies used are given in S1 Table.

Statistics

Differences between *two groups* for single point data were determined by Student's *t*-test. All numerical data are represented as mean ± SE. Significant difference was set at $P < 0.05$.

Results

BA-exposure improves metabolic abnormalities induced by HFD

In order to understand the potential impact on male fertility of long term treatment of MetS diseases with molecules that activate bile acid signaling pathways, male mice were exposed to high fat diet (HFD) and then to HFD supplemented with 0.5% cholic acid (HF-CA) as previously described by Watanabe et al. [7]. Male mice exposed to HFD showed increased body

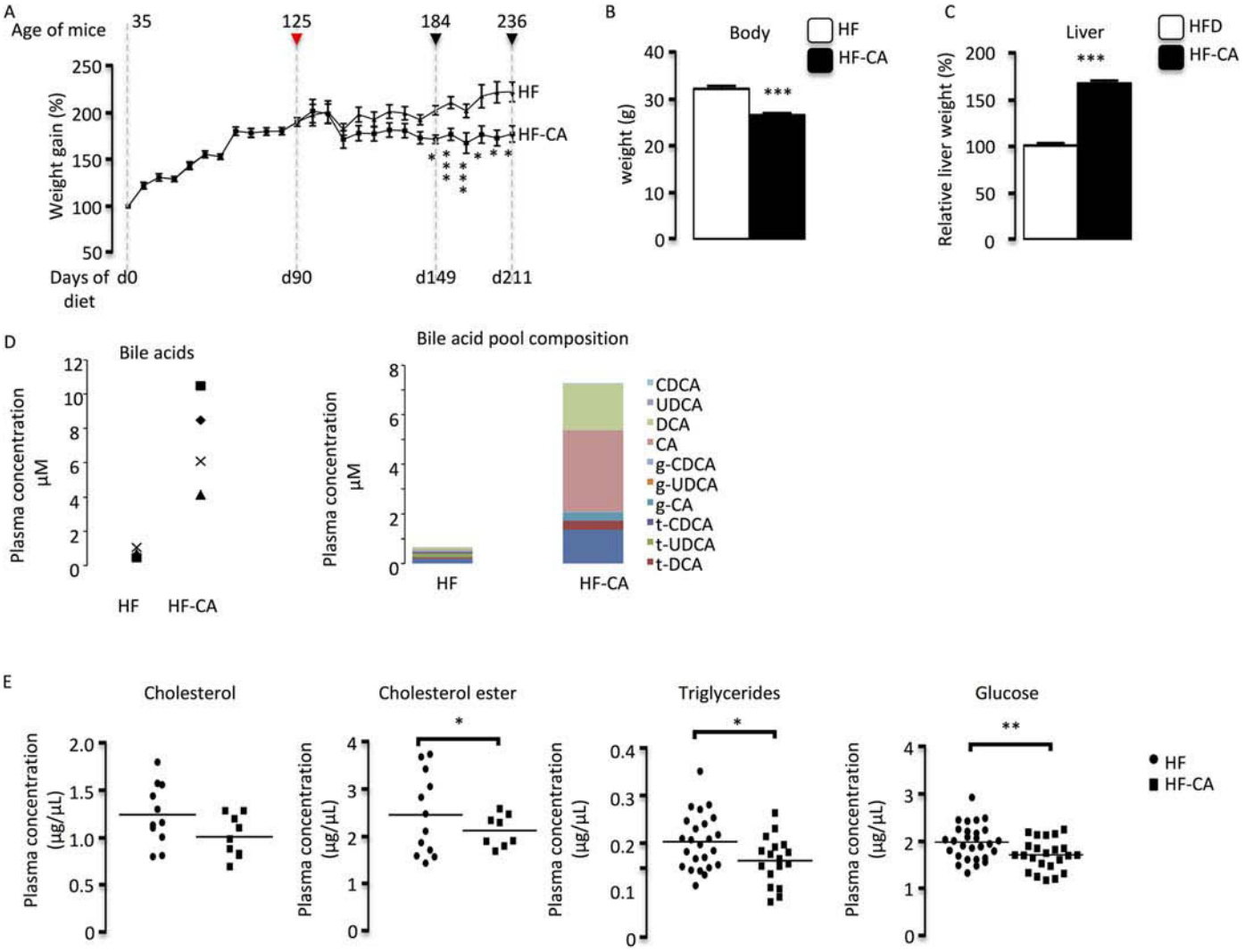

Fig 1. CA-supplementation reverse HFD induced overweight. (A) Weight gain of C57BL/6J mice along the experiments. After 90 days of high fat diet (HFD) (red arrow), half of the mice on the HFD (triangles) were switched to HFD supplemented with CA (HF-CA) (squares) (n = 12–35 per group). Black arrows indicated the timing of fertility test. **(B)** Relative body weight 4 months after the switch to HF-CA diet. **(C)** Relative liver weight normalized to body weight in C57Bl/6 mice fed HFD and HF-CA diet 4 months after the switch to HF-CA diet. (n = 18–25 per group). **(D)** Plasma bile acid levels and pool composition in mice under HFD or HF-CA diet 4 months after the switch to HF-CA diet. **(D)** Plasma cholesterol, cholesterol ester, triglycerides and glucose levels in mice fed to HFD or HF-CA diets 4 months after the switch to HF-CA diet. (n = 19–25 per group). Data represent mean ± SEM; Statistical analyses: * p<0,05; ** p<0,01 and *** p<0,001.

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weight superior to animal under chow diet throughout the experiment (S1C Fig), validating our model to induce mouse overweight. In addition, HFD induced increased levels of cholesterol, cholesterol ester, and glucose (S1D Fig). Once the overweight is established, part of the mice fed HFD was shifted to HF-CA to reverse the obesity. As expected, these mice showed a decreased of body weight gain compared to HFD group that is significant at 2 and 4 months after the switch to HF-CA diet (S1D Fig and Fig 1A & 1B). In accordance with bile acid effects [5], liver weight was increased in BA-exposed group compared to HFD group since 2 months after diet switch (S1E Fig) and still increased after 4 months (Fig 1C). Plasma BA levels were highly increased in HF-CA group compared to HFD group (Fig 1D). The analysis of the BA

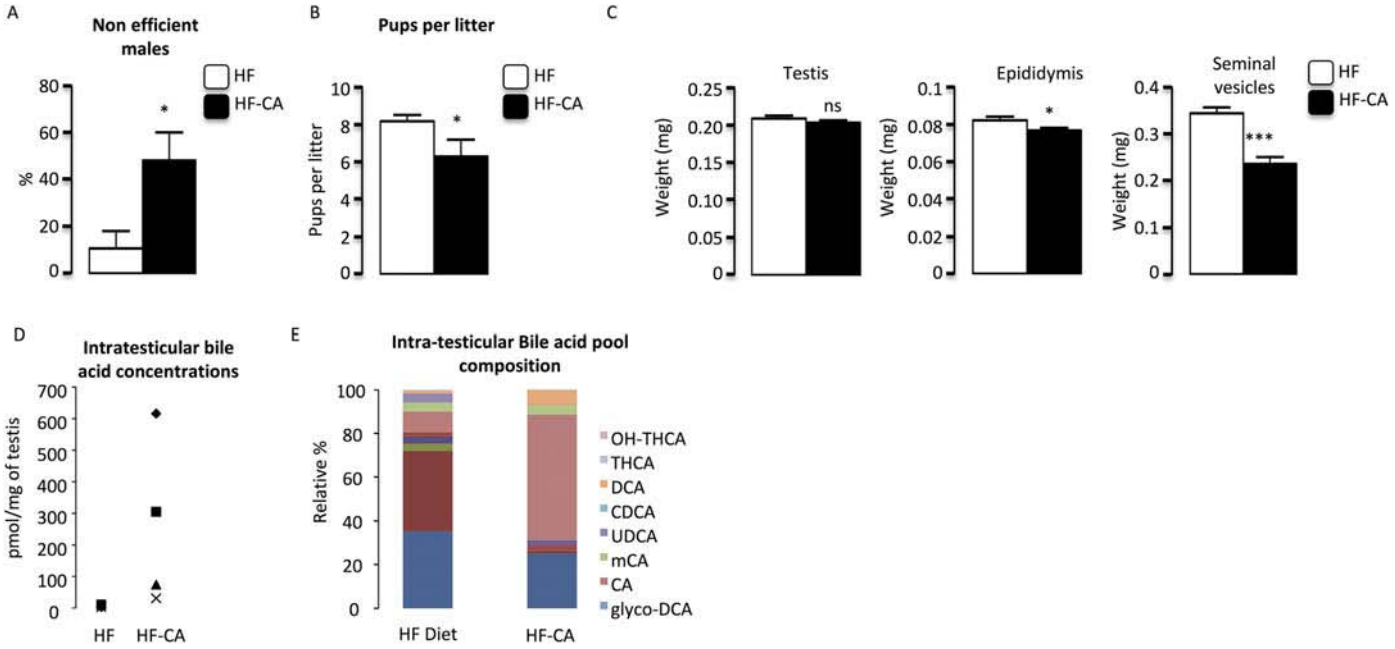

Fig 2. CA-supplementation alters male reproduction function in mice fed HF-CA diets versus HF-diet. (A) Percent of non-efficient males after 15 days of breeding with 2 C57BL/6J females in mice under HFD or HF-CA diet 4 months after the switch to HF-CA diet. **(B)** Number of pups per litter obtained in mice under HFD or HF-CA diet 4 months after the switch to HF-CA diet. **(C)** Relative testis, epididymis and seminal weights normalized to body weight in C57Bl/6 mice fed HF-diet and HF-CA diet 4 months after the switch to HF-CA diet. (n = 18–25 per group). **(D)** Intra-testicular bile acid levels and pool composition in mice under HFD or HF-CA diet 4 months after the switch to HF-CA diet. Data represent mean ± SEM; Statistical analyses: * p<0,05; ** p<0,01 and *** p<0,001.

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pool revealed the increase concentrations of CA and DCA species (Fig 1D). As expected [4], HF-CA diet led to a decrease of plasma total cholesterol and cholesterol esters and triglyceride levels (Fig 1E). Accordingly, the impact of the HF-CA diet was confirmed at the glucose levels which were lower compared to HFD group (Fig 1E). Note that for some of these plasma parameters effects were observed since 2 months after the switch to HF-CA diet (S2A Fig).

BA-exposure alters male fertility in the context of metabolic syndrome

As BA exposure was previously demonstrated to induce male fertility disorders, we next analyzed male reproductive capacity. Male fertility was assessed by breeding males of each group with C57Bl6J females during 2 weeks (as described in method section). Fertility tests highlight reproductive defaults in HF-CA group compared to HFD group (Fig 2A). Even if there was no difference in the number of vaginal plugs observed per male between groups (data not shown), data revealed a 4 to 5-fold increase in the number of males unable to give progeny after two weeks of breeding with females; these males were thus qualified as non-efficient males (Fig 2A). For the males that were still able to give progenies, there was a significant decrease in the number of pups per litter in HF-CA animals (Fig 2B). No significant effect of CA-supplementation was observed on fertility parameters 2 months after the switch to HF-CA diet (S2B Fig).

This impact on fertility was sustained by lower weight of organs of genital tract such as epididymis and seminals in HF-CA treated males compared to HFD group (Fig 2C **and** S2C Fig). Note that no effect was observed on testis weight (Fig 2C).

We then analyzed whether bile acid levels and pool were modified in HF-CA group compared to HFD males. We show a 6-fold increase of BA concentrations in testis of males exposed

to HF-CA diet compared to HFD group (Fig 2D). This is consistent with what was previously published by Baptissart et al. [5]. Consistently, BA pool analyses revealed the increase concentration of CA and DCA species (Fig 2E).

BAs induce lower germ cell proliferation rate

HF-CA diet did not drastically alter histology of testis compared to HFD group (data not shown). No statistical impact of HF-CA-diet was observed on germ cell apoptosis compared to HFD-group (Fig 3A). However, we confirmed that as previously described HFD induced germ

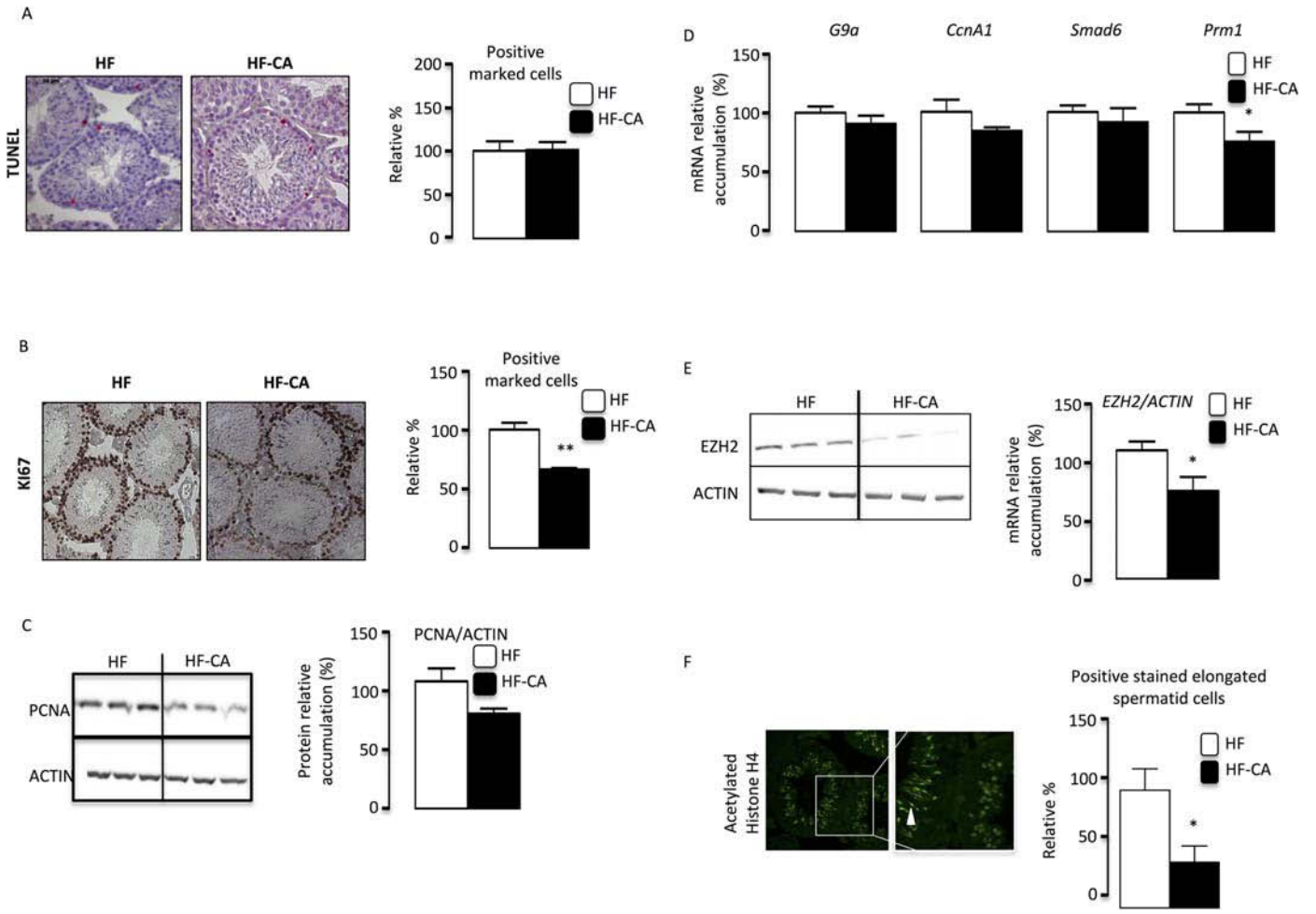

Fig 3. CA-supplementation diet induces increase of germ cell proliferation. (A) Apoptosis in mice exposed to HFD or HF-CA diets 4 months after the switch to HF-CA diet (n = 13 to 25 per group) analyzed by TUNEL staining. The arrow indicates apoptotic spermatocytes. The original magnification was X200. The number of TUNEL-positive cells per 100 seminiferous tubes. **(B)** Proliferation in mice exposed to HFD or HF-CA diets (n = per groups) analyzed by Ki–67 staining. Representative micrographs of the testis exposed to HF and HF-CA diets. The original magnification was X100.Quantification of the number of Ki-67-positive cells per 100 seminiferous tubules after 2 and 4 months of HF and HF-CA diets (n = 4–5 per groups). **(C)** Immunoblot of PCNA and ACTIN on testicular protein extracts of HFD or HF-CA diet 4 months after the switch to HF-CA diet (n = 5–8 per groups). Quantification of PCNA/ACTIN ratio. HFD group was arbitrarily fixed at 100%. **(D)** Testicular mRNA expression of *G9a*, *Cyclina1 (Ccna1)*, *Smad6* and *Prm1* normalize to β-actin levels in whole testis of C57BL/6 mice fed HFD or HF-CA diets 4 months after the switch to HF-CA diet (n = 16–22 per groups).). **(E)** Immunoblot of EZH2 and ACTIN on testicular protein extracts of HF diets or HF-CA diets 4 months after the switch to HF-CA diet (n = 5–8 per groups). Quantification of EZH2/ACTIN ratio. HFD group was arbitrarily fixed at 100%. **(F)** Evaluation of post-meiotic elongated spermatids in mice exposed to HFD or HF-CA diets analyzed by acetylated histone H4 (H4ac) staining 4 months after the switch to HF-CA (Representative micrographs of the testis exposed to HF diet). The original magnification was X100.Quantification of the number of H4ac-positive cells (n = 4–5 per groups). Data represent mean ± SEM; Statistical analyses: * p<0,05; ** p<0,01 and *** p<0,005.

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cell apoptosis (S3A Fig). In contrast, a significant decrease of germ cell proliferation was observed at 4 months in HF-CA group compared to HFD-group as identified by Ki67 staining (Fig 3B). This decrease in germ cell proliferation was supported by the lower accumulation of the PCNA protein in HF-CA group compared to HFD animals (Fig 3C). This data suggests that there could be some abnormalities in spermatogenesis process. In order to define which spermatogenesis step might be altered, we performed analysis of mRNA accumulation of specific markers for early spermatogenesis (G9a), spermatocytes (Cyclin-a1), round spermatids (Smad–6) and late germ cells (Protamin–1; Prm–1). Results show a lower expression of *Prm–1* a gene referred to be expressed in elongating/elongated spermatids (Fig 3D). This is also sustained by the lower EZH2 protein level, a marker of post-meiotic germ cells [12], in HF-CA group compared to HFD animals (Fig 3E). The decrease of number of post-meiotic cells within the testis exposed to HF-CA diet was then validated by immunohistochesmistry with acetylated-histone H4 staining and the identification of a lower the number of positive cells per tubes in HF-CA group compared to HFD exposed males (Fig 3F). No difference was observed regarding intra-testicular levels of some lipid parameters such as cholesterol, cholesterol ester or triglycerides either 2 or 4 months after the switch to HF-CA diet (S3B and S3C Fig).

BA exposure leads to altered testicular endocrine function

Testicular physiology has been demonstrated to be highly dependent of steroid metabolism as highlighted by the numerous data coming from studies on endocrine disrupters. 4 months after the switch to HF-CA diet; CA supplementation alters testicular steroidogenesis as highlighted by the lower intra-testicular and plasma testosterone levels in HF-CA diet group compared to HFD group (Fig 4A & 4B). Interestingly, FXRα has been previously demonstrated to repress testicular steroidogenesis via the induction of SHP which in turn inhibits *Sf–1* and *Lrh–1* expression and their transcriptional activities [6], [13]. The potential activation of the FXRα signaling pathways within the testis in the HF-CA context was assessed through the analysis of FXRα and two of its target genes *Shp* and *Bsep*. Shp was not affected after 4 months of diet (Fig 4E). However, we found an increase of FXRα and Shp mRNA accumulation if HF-CA group compare to HFD group after two months of HF-CA diet exposure (Fig 4G). In contrast, a lower mRNA accumulation of *Bsep* was observed after two months of HF-CA diet exposure (Fig 4G). This downregulation of *Bsep* must be correlated to the higher level of Shp as it was previously demonstrated that SHP inhibit the expression of *Bsep* through LRH–1 dependent activity [6]. The analysis of *Sf–1* and *Lrh–1* revealed that their expression levels were not affected in HF-CA exposed group compared to HFD animals at 4 months or 2 months after the beginning of HF-CA diet (Fig 4E & 4G). These data suggest that SHP might act mainly through the inhibition of the transcriptional activity of LRH–1 and SF–1 as previously suggested in some recent reports [8]. Even though short term experiments are need to clearly characterize the induction of FXRα pathways in the HF-CA context, we decided to explore the impact of HF-CA on testicular steroidogenesis. Indeed, regarding the testicular physiology, the only define pathway targeted by FXRα so far is the steroidogenesis. This effect was published to be dependent of SHP [6]. In that line, we define that after two months of HF-CA diet the expression of steroidogenic gene *Cyp11a1* was decreased (Fig 4G). In addition the decreased testicular testosterone levels after 4 months of HF-CA diet was correlated with the impact on the mRNA accumulation of genes involved in steroid synthesis such as *Star*, *Cyp11a1* and *Cyp17a1* (Fig 4C). Furthermore, these results were associated with decreased expression levels of several androgen-dependent genes (*Tubb3*, *Atp1a2*, *Pem*) four months after the beginning of HF-CA diet (Fig 4D). This impact on androgen-dependent genes was not due to alteration of the expression of the androgen receptor neither at the mRNA level (Fig 4E) nor at the protein level (Fig 4E).

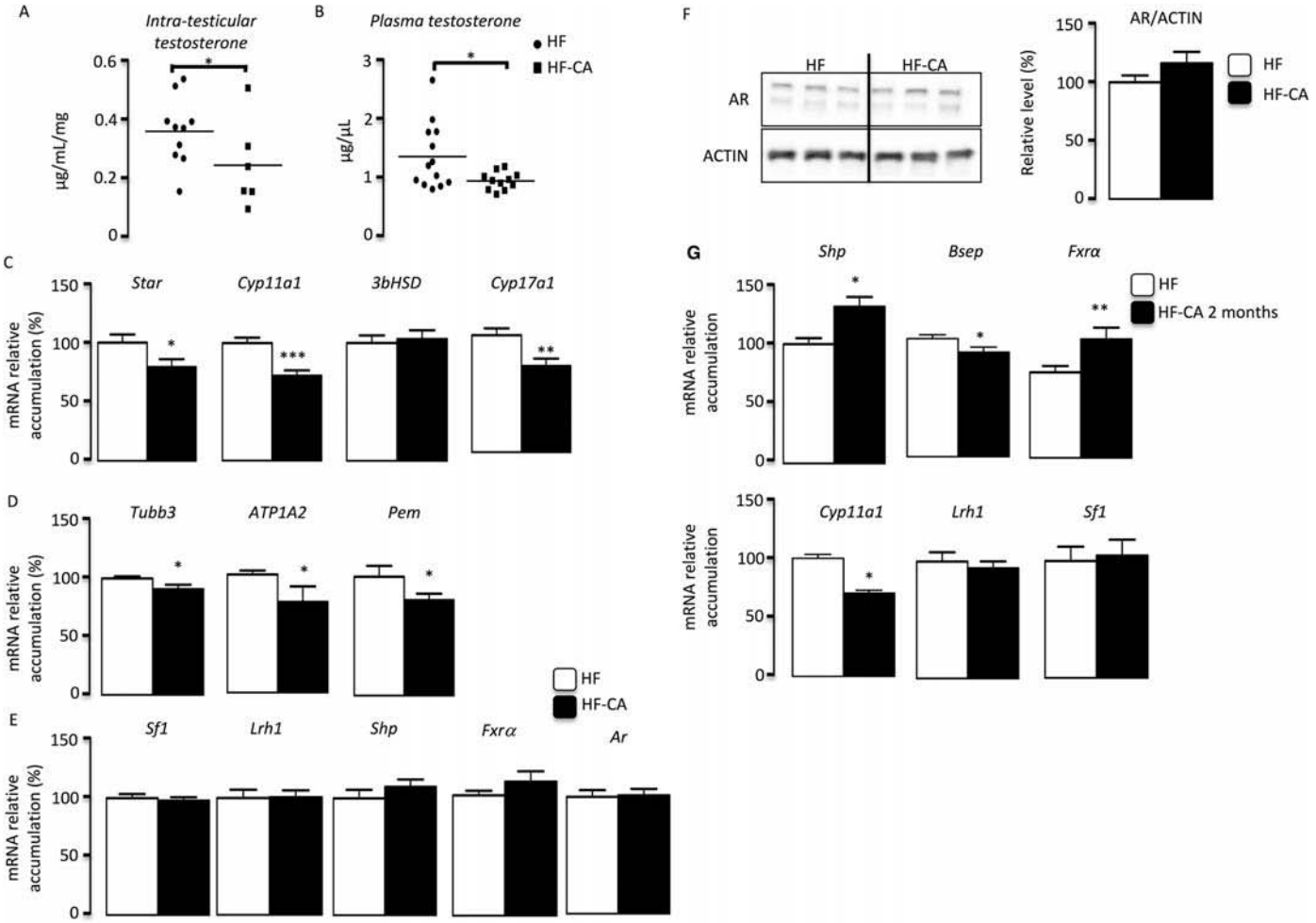

Fig 4. CA-supplementation alters testicular endocrine function. (A) Testicular testosterone levels in mice fed HFD and HF-CA diet 4 months after the switch to HF-CA diet. (n = 7–13 per group). **(B)** Plasma testosterone levels in mice fed HFD and HF-CA diet 4 months after the switch to HF-CA diet. (n = 7–13 per group).**(C)** Testicular mRNA accumulation of *Star*, *Cyp11a1*, *3βHDS* and *Cyp17a1* normalized to *β-actin* mRNA levels in the whole testes of mice fed HFD and HF-CA diet 4 months after the switch to HF-CA diet (n = 12–22 per group). **(D)** Testicular mRNA expression of *Tubb3*, *Atp1a2* and *Pem* normalized to *βactin* m in the whole testes of mice fed HFD and HF-CA diet 4 months after the switch to HF-CA diet (n = 7–22 per group). **(E)** Testicular mRNA expression of *Sf–1*, *Lrh–1*, *Shp*, *Fxrα* and *Ar* normalized to *β-actin* m in the whole testes of mice fed HFD and HF-CA diet 4 months after the switch to HF-CA diet (n = 7–22 per group). **(F)** Immunoblot of AR and ACTIN on testicular protein extracts of HFD or HF-CA diet 4 months after the switch to HF-CA diet (n = 5–8 per groups). Quantification of AR/ACTIN ratio. HF-diet group was arbitrarily fixed at 100%.HF-diet group was arbitrarily fixed at 100%. **(G)**. Testicular mRNA expression of *Shp*, *Bsep*, *Fxrα*, *Cyp11a1*, *Lrh–1*, Sf–1 and normalize to β-actin levels in whole testis of C57BL/6 mice fed HFD or HF-CA diet 2 months after the switch to HF-CA diet (n = 16–22 per groups. Data represent mean ± SEM; Statistical analyses: * p<0,05; ** p<0,01 and *** p<0,005.

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BAs alter seminiferous epithelium integrity

We have recently identified that BA impact testicular physiology through TGR5 dependent mechanisms [5]. TGR5 activation leads to the control of the TBX2-Cx43 pathways which is associated with the alteration of the blood-testis-barrier (BTB). The BTB is a critical structure of the seminiferous epithelium and has been demonstrated to be altered following the increase of BA levels. Here, we show that the BTB is no longer intact after 4 months of HF-CA diet, as shown by the use of biotin-coupled tracer (Fig 5A). Consistent with what was previously published, bile acid exposure decreased accumulation of *Connexin–43* at both mRNA and protein (Fig 5B & 5C). This was associated with the increase of *Tbx2* mRNA accumulation in HF-CA

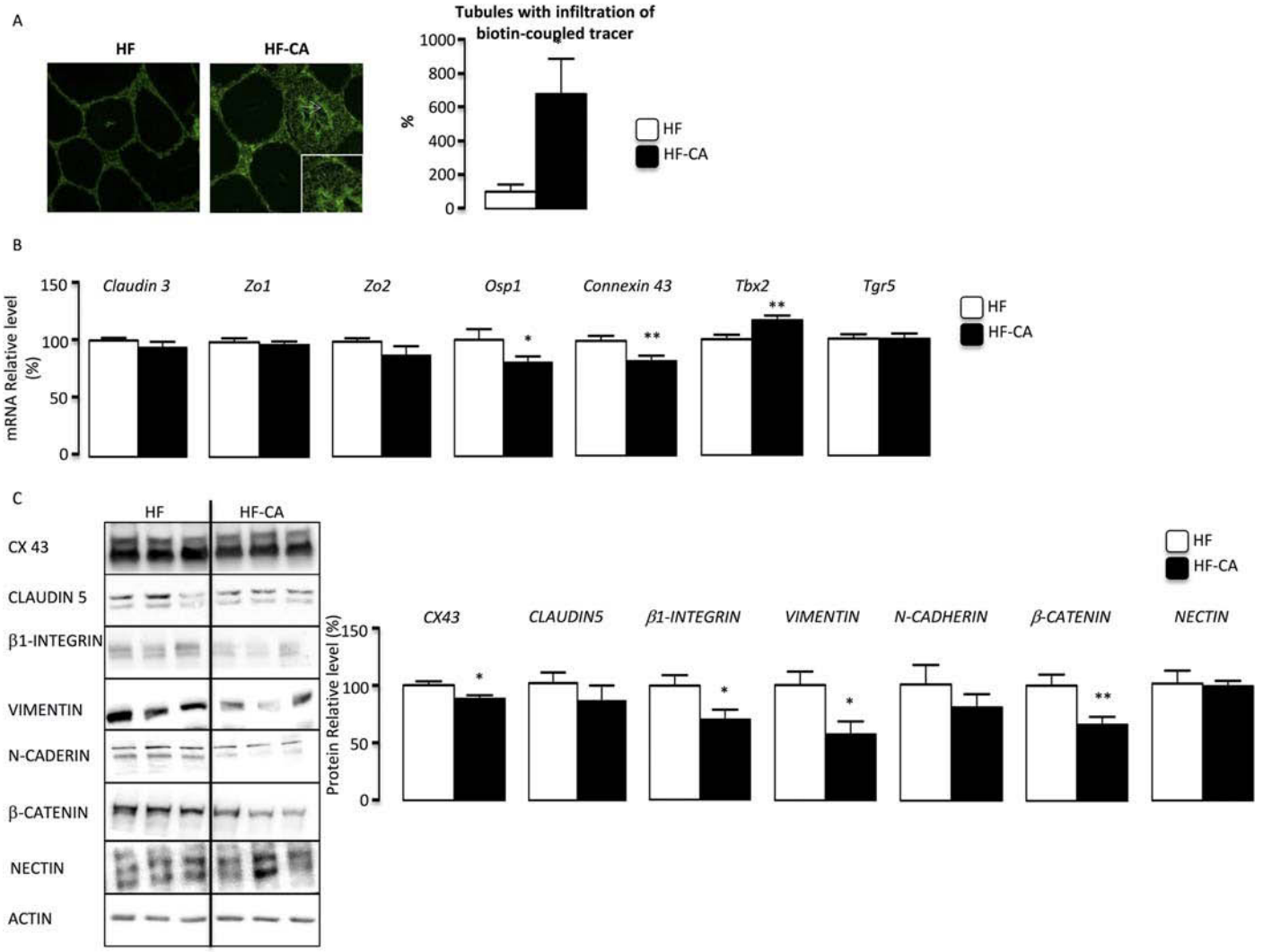

Fig 5. CA-supplementation alters seminiferous epithelium. (A) Blood-testis barrier integrity as measured by the stained testis for EZ-link-biotinylated. Representative micrographs of mice fed HFD or HF-CA diet 4 months after the switch to HF-CA diet. The arrow indicates a tubule with a high intensity of infiltration. The original magnification was X100. Quantification of the number of tubules with infiltration per 100 seminiferous tubules (n = 5–12 per group). **(B)** Testicular mRNA expression of *Claudin–3*, *Zo1*, *Zo2*, Osp1,*Cx 43*, *Tbx2 and Tgr5* normalize to β-actin levels in whole testis of C57BL/6 mice fed HFD or HF-CA diet 4 months after the switch to HF-CA diet (n = 16–22 per groups). **(C)** Immunoblot of CONNEXIN43, CLAUDIN5, INTEGRINβ1, VIMENTIN, N-CADHERINE, β-CATENINE and NECTIN on testicular protein extracts of HFD or HF-CA diet for 4 months (n = 5–8 per groups). Quantifications of proteins of interest were made normalized to ACTIN. HF-diet group was arbitrarily fixed at 100%. Data represent mean ± SEM; Statistical analyses: * p<0,05; ** p<0,01 and *** p<0,005.

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group compared to HFD after 4 months of diet exposure (Fig 5B). These data suggest that TGR5 pathways might have been induced by CA-supplementation. However, no effect of HF-CA diet was observed on TGR5 mRNA expression (Fig 5B). Regarding the expression of other genes involved in cell-cell interactions, a lower mRNA accumulation of *Osp1* in BA-fed mice was observed (Fig 5B). In contrast, no effects were observed regarding the mRNA accumulation of *Zo1*, *Zo2* and *Claudin3* (Fig 5C). No effect was observed on CLAUDIN–5, NECTIN protein accumulations (Fig 5C); however we confirmed the impact of cell-cell interactions within the seminiferous tubule as the protein accumulations of INTEGRIN-β1, VIMENTINE,

N-CADHERIN and β-CATENINE was decreased in HF-CA exposed males compare to HFD group (Fig 5C).

Discussion

Recently, we showed that adult mice fed a diet supplemented with CA have altered fertility subsequent to testicular defects [5]. The use of BA supplementation has been demonstrated to be able to reverse obesity induced by HFD in mice. In the following years, subsequent studies highlighted the links between BA exposure and improvement of diseases such as diabetes.

We thus wonder what could be the consequences on male fertility of a long term exposure to BA derivatives in the treatment of MetS. To investigate these potential links, we have reproduced the protocol of Watanabe and collaborators [7]. Male mice were exposed to HFD to induce overweight and use supplementation with 0.5% cholic acid to reverse the obesity.

The present work confirms the deleterious impact of BA exposure on male fertility even during the treatment of MetS. Indeed, the BA-supplemented diet led to an increase of BA concentrations in plasma and testis. The composition of BA species within the testis in HF-CA exposed males are comparable to what was previously published by Baptissartwith the increase of CA and DCA species.

The complexity of the interactions between metabolism and male fertility has been described among the last decades [14]. Among all the consequences of MetS, male reproductive function is altered in some obese man patients. This is highlighted by the increasing evidence of an interactions between MetS and testicular functions [14]. However, the relation between MetS and male reproductive function remains to be fully deciphered.

This complexity still need to be highly studied as we demonstrate here that even with the amelioration of several global health parameters such as plasma cholesterol, triglycerides and glucose male fertility remain altered. In that line BA-diet have no impact on metabolic parameters (glucose, cholesterol, triglycerides) in the testis, it reverse the impact of HF diet on these parameters at the plasma level. Thus our data reinforce the links between BA signaling pathways and testicular physiology and pathophysiology. However, it could not be excluded that their might be direct impact of BA-exposure on organs involved in post-testicular maturation of the spermatozoa such as the epididymis and the seminals. Such effects should be studied in future works as the decrease of seminal weights under BA-diet conditions are reported here and in previous report [5].

Even though the clear involvement of systemic or local testicular action of BA receptors cannot be established there as specific testicular invalidation would have been required, we demonstrate that BA exposure impact testicular physiology in HF-CA context. Our goal here was to define if alterations induced by BA-exposure, as previously published, are reproducible in HF context.

Previous report highlights the major role of TGR5 regarding the impact of BA on male fertility during cholestasis. The results of the present study supported the activation of the TGR5 signaling pathways in testis following HF-CA diet exposure as we do observed as previously published [5] the increase of *Tbx2* mRNA accumulation and the decrease of CX43 at both mRNA and protein levels.

However, the present work clearly suggests that in the context of MetS, the involved mechanisms are more complex than previously describe. Indeed, in contrast to the study from Baptissart et al., HF-CA diet leads to a deregulation of testicular steroidogenesis. Such effect could be consistent to some of the effect of the FXRα signaling pathway within the testis as the use of GW4064, a FXRαsynthetic agonist, was demonstrated to repress testosterone synthesis in a SHP dependent manner [6]. In that line we do observed that after HF-CA administration the

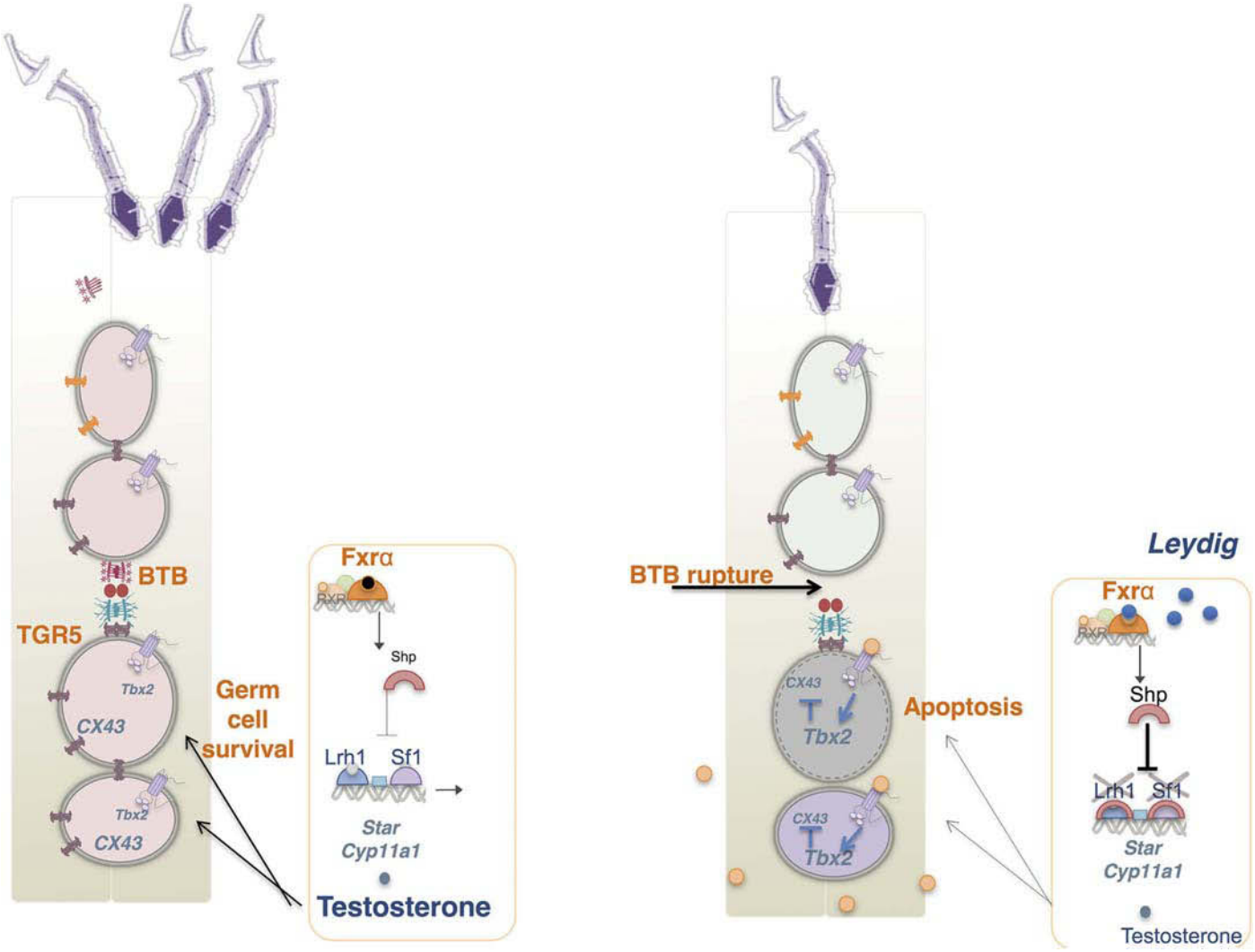

Fig 6. Potential intratesticular action of BAs. In normal condition production of testosterone is involved in germ cell survival. The expression of genes involved in steroidogenic pathway is in part supported by transcriptional activity of LRH–1 and SF1. In parallel, the integrity of the seminiferous epithelium is ensured by the establishment of cell-cell interactions involving protein such as Cx43. In the context of BA exposure, lower production of testosterone is observed. This is consistent with the potential activation of the FXRαwhich in turn leads to activation of SHP a known repressor of steroidogenic pathways via the inhibition of transcriptional activity of LRH–1 and SF–1 on promoter sequences of genes such as Star or Cyp11a1. In parallel, in BA context, the integrity of blood testis barrier is altered. This is consistent with the activation of the TGR5-Tbx2 signaling pathways leading to lower accumulation of protein involved in cell-cell interactions that destabilized the structure of the seminiferous epithelium. These alterations might participate to the increase rate of germ cell apoptosis within the testis. In regards to the major role of testicular physiology, even though post-testicular impact cannot be exclude; the present work suggests that this alterations of testicular physiology induced in a HF-diet context might participate to the appearance of male infertility.

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expression of SHP was increased with a decrease of steroidogenic genes and then testosterone levels. In addition the lower mRNA accumulation of *Bsep* was observed after two months of HF-CA diet exposure (Fig 4G). This downregulation of *Bsep* must be correlated to the higher level of Shp as it was previously demonstrated that SHP inhibit the expression of *Bsep* through LRH–1 dependent activity [6].

Here, no difference in the status of germ cell apoptosis was observed between HFD and HF-CA groups; this is in contrast with previous data showing apoptosis of post-meiotic germ cells (spermatids) during liver disease induced by BA exposure [5]. This might be due to the

fact that HFD *per se* induces apoptosis of spermatocytes cells (S3A Fig), which are earlier germ cell steps than spermatids, this effect of HFD might mask the impact of BA on spermatids. However, a lower production of post-meiotic germ cells in HF-CA group is sustained by our results showing lower accumulation of post-meiotic germ cells markers at either mRNA and protein levels.

The present work suggests that in the pathophysiological model of HF-diet, several BA signaling pathways among which either FXRα or TGR5 could be activated. Additional studies must be performed using specific agonists of FXRα and TGR5 to better decipher the critical pathways involved. Using an integrative scheme, we try here to summarize the potential involvement of both signaling pathways within the testis (Fig 6).

In addition, the present results on the impact of BA exposure in context of HFD on fertility could be useful to understand some unexplained rare clinical situations. This could be consistent with a case report data showing subsequent infertility in some obese men treated with bariatric chirurgical approach [15]. It is of interest to note that in such treatment, BA levels have been demonstrated to be increased [16]. Even if it is with low incidence, our work could open new perspectives to better understand such rare pathological cases.

This work highlights the urgent need to clearly define the molecular mechanisms driving the deleterious impact of BAs on testis. Further studies will be necessary to better define the pharmacologic or genetic modulations of different bile acid receptors during the treatment of metabolic disorders in order to minimize the impact on male reproductive functions.

In conclusion, our study raises the question of the long term consequences of treatment with BA derivatives. Thus it will be necessary to plan future experiments to define the potential secondary impacts of the activation of BA signaling pathway as the use of derivates from BAs as therapeutic molecules are proposed today (metabolic syndrome, cancer).

Supporting Information

S1 ARRIVE Checklist. NC3Rs ARRIVE Guidelines Checklist. The ARRIVE guideline checklist for animal research: reporting in vivo experiments.
(DOCX)

S1 Fig. (A). Quantification of the number of seminiferous tubules per testis slide in HFD and HF-CA fed animals 4 months after the switch to HF-CA diet. (B). Quantification of the diameter of seminiferous tubules per testis slide in HFD and HF-CA fed animals 4 months after the switch to HF-CA diet. (C) Weight gain of C57BL/6J mice along the experiments fed chow or HFD for 236 days. (D) Relative body weight 2 months after the switch to HF-CA diet. (E) Relative liver weight normalized to body weight in C57Bl/6 mice fed HFD and HF-CA diet 2 months after the switch to HF-CA diet. (n = 18–25 per group). (E) Plasma cholesterol, cholesterol ester and glucose levels in mice fed to chow or HFD (n = 19–25 per group). Data represent mean ± SEM; Statistical analyses: * $p<0,05$; ** $p<0,01$ and *** $p<0,001$.
(PDF)

S2 Fig. (A). Plasma cholesterol, cholesterol ester, triglycerides and glucose levels in mice fed to HFD or HF-CA diets 2 months after the switch to HF-CA diet. (n = 19–25 per group). (B) Percent of non-efficient males after 15 days of breeding with 2 C57BL/6J females to analyse their capacity to mate. (C) Number of pups per litter obtained. (D) Relative testis, epididymis and seminal weights normalized to body weight in C57Bl/6 mice fed HFD and HF-CA diet 2 months after the switch to HF-CA diet. (n = 18–25 per group). Data represent mean ± SEM; Statistical analyses: * $p<0,05$; ** $p<0,01$ and *** $p<0,001$.
(PDF)

S3 Fig. (A) Apoptosis in mice fed chow or HFD (n = 13 to 25 per group) analyzed by TUNEL staining. The arrow indicates apoptotic spermatocytes. The original magnification was X200. The number of TUNEL-positive cells per 100 seminiferous tubes. (B) Intra-testicular cholesterol, cholesterol ester, triglycerides levels in mice fed to HFD or HF-CA diet 2 months after the switch to HF-CA diet. (n = 19–25 per group). (C) Intra-testicular cholesterol, cholesterol ester, triglycerides and glucose levels in mice fed to HFD or HF-CA diet 4 months after the switch to HF-CA diet. (n = 19–25 per group).
(PDF)

S1 Table. The list of the antibodies used in the present work.
(XLSX)

S2 Table. The list of the primer sequences used in the present work.
(XLSX)

Author Contributions

Conceived and designed the experiments: DHV. Performed the experiments: AV EM MB ADH FV WK CDS. Analyzed the data: AV EM MB SB FV WK FC DHV. Contributed reagents/materials/analysis tools: SB DHV. Wrote the paper: AV DHV.

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Résumé : Etude du rôle du récepteur nucléaire FXRα dans la physiologie et la physiopathologie testiculaire.

Fxrα est le récepteur nucléaire des acides biliaires, exprimé majoritairement dans le foie, l'intestin, les reins et les glandes surrénales. L'intérêt pour ce dernier est devenu croissant au cours des dernières années, de part le rôle central qu'il joue dans le contrôle de l'homéostasie du cholestérol, des acides biliaires, des triglycérides ou encore du glucose.

Plus récemment, Fxrα ainsi que ses ligands, les acides biliaires, ont été localisés dans le testicule, soulevant la question du rôle potentiel de Fxrα dans cet organe, et plus généralement dans la fonction de reproduction mâle. Mais les études menées à ce sujet restent jusqu'à présent peu nombreuses, et focalisées sur son implication dans le contrôle du métabolisme des stéroïdes : l'activation *in vivo* de Fxrα par un agoniste synthétique conduit ainsi chez l'adulte à court terme à une répression de la stéroïdogenèse.

Outre son rôle dans le contrôle de l'activité endocrine des cellules de Leydig, l'impact de l'activation *in vivo* de Fxrα sur la physiologie plus globale du testicule n'a jamais été abordé à ce jour. De telles études seraient pourtant pertinentes étant donné que Fxrα est ciblé pour le traitement de pathologies métaboliques telles que la dyslipidémie ou le diabète.

Dans ce contexte, l'objectif de ce travail de thèse était d'étudier le rôle de Fxrα dans la physiologie et la patho-physiologie du testicule, en s'appuyant sur l'analyse d'un modèle murin dont le gène codant Fxrα a été invalidé. Nos résultats démontrent que : 1) la perte de Fxrα prédispose le testicule à une sur-mortalité des cellules germinales dans un contexte pathologique de cholestase ; 2) la sur-activation de la signalisation Fxrα au cours de la puberté conduit à un défaut de la différenciation germinale, associée à une altération de la fonction endocrine du testicule ; 3) outre la régulation de la stéroïdogenèse dans les cellules de Leydig, Fxrα participe au contrôle des fonctions sertoliennes et de la prolifération et / ou différenciation des cellules germinales souches.

L'ensemble de ces données définissent Fxrα comme un nouvel acteur impliqué dans le contrôle de la physiologie testiculaire et devraient être prises en considération quant-à l'utilisation de molécules agonistes et / ou antagonistes de Fxrα dans le cadre du traitement de pathologies métaboliques.

Mots clés : Fxrα, testicule, stéroïdogenèse, apoptose et différenciation germinale, cellules germinales souches.

Abstract : Role of the nuclear receptor FXRα in testicular physiology and pathophysiology.

Fxrα is the bile acid nuclear receptor, predominantly expressed in liver, intestine, kidney and adrenal glands. In recent years, interest in Fxrα has been increasing due to its central role in the control of cholesterol, bile acids, triglycerides or glucose homeostasis.

More recently, Fxrα and its ligands, bile acids, have been detected in the testis pointing out its potential involvement in this tissue and more widely in the male reproductive functions. However, the few studies on this topic focused essentially on Fxrα involvement in the control of steroids metabolism. Indeed, activation of Fxrα *in vivo* with a synthetic agonist leads to short-term steroidogenesis repression in the adult.

In vivo the impact of alteration of Fxrα signaling on the global testis physiology has never been explored so far. Such studies would be pertinent considering that Fxrα is a target for the treatment of metabolic diseases such as dyslipidemia or diabetes.

In this context, the aim of my work was to study the implication of Fxrα in testis physiology and physiopathology by analyzing a knock out mouse model for Fxrα. Our results show that: 1) the loss of Fxrα increase germ cell mortality in the testis in a disease context of cholestasis ; 2) over-activation of Fxrα signaling during puberty leads to germ cell differentiation defects, associated with an alteration of testis endocrine function ; 3) besides steroidogenesis control in Leydig cell, Fxrα is involved in Sertoli cell functions and spermatogonial stem cell proliferation and/or differentiation.

Taken together, these data define Fxrα as a new actor involved in the control of testis physiology, and should be taken into consideration regarding the use of Fxrα agonistic or antagonistic ligands for the treatment of metabolic diseases.

Key words : Fxrα, testis, steroidogenesis, germ cell apoptosis and differenciation, spermatogonial stem cells.